

General Structure of Heterobifunctional Linkers



R = Alkyl, cycloalkyl, cycloalkyl-alkyl, aromatic, alkyl-aromatic, stilbene, heterocyclic, alkyl-heterocyclic, CH₂CH₂-O-, alkyl-CH₂CH₂-O-alkyl, CH₂-CH=CH-, CH₂-NHCO, alkyl-NHCO-alkyl, CH₂CH₂-S-, CH₂CH₂-NH-, Long Chain Alkyl Amino, etc.

X = NH₂, succinimidyl, maleimidyl, iodoacetamido, bromoacetamido, thiol,

Y = Biotin

= Biotin/Avidin

= Biotin/Streptavidin (SA)

= Alkaline Phosphatase (AP)

= Casein

= beta-Lactamase

= BSA

= IgG

= Avidin-AP

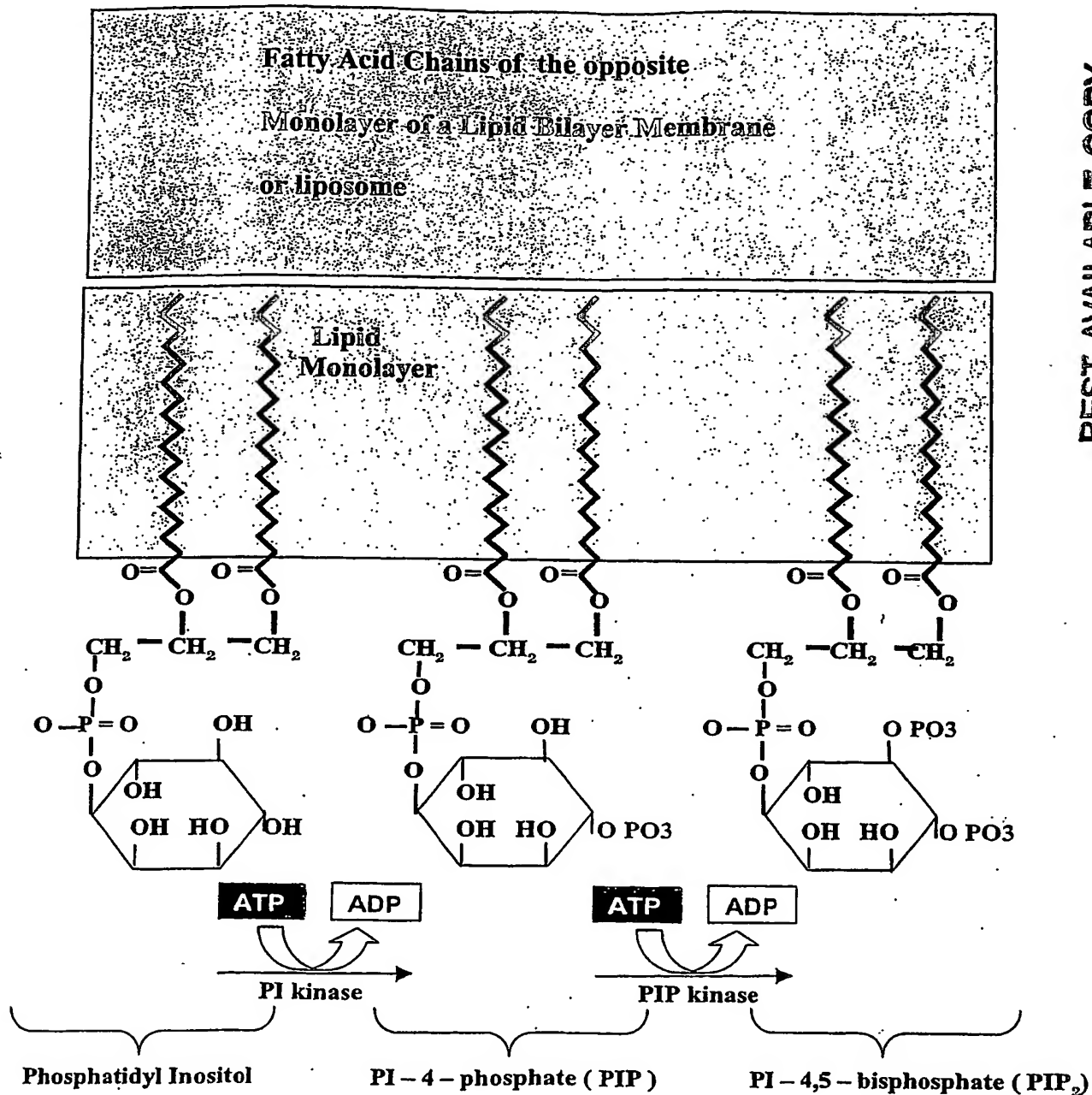
= Streptavidin-AP

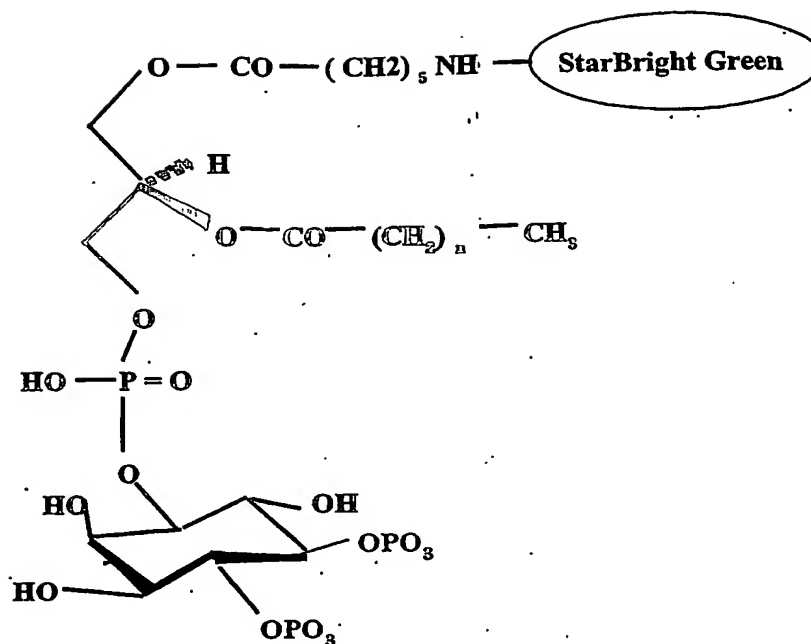
= Biotin or Streptavidin complexed with :

Glycoproteins, enzymes, antibodies, DNA, RNA, peptides, derivatized particles made of polystyrene, nylon, gold, polyacrylamide, and other solid surfaces such as microtitre plates, glass (silicon) plates, and any other polymer comprised of active functions, for example, -OH, -NH₂, -SH, succinimidyl, maleimido groups.

Figure 1. General chemical structure and compositions of the heterobifunctional linkers of the Present Invention

Figure 2. Classification of Kinases and Phosphatases by Target Structure





STARBRIGHT GREEN - PHOSPHATIDYLINOSITOL- 4,5- BISPHOSPHATE
[STARBRIGHT GREEN - PtdIns(4,5)P2]

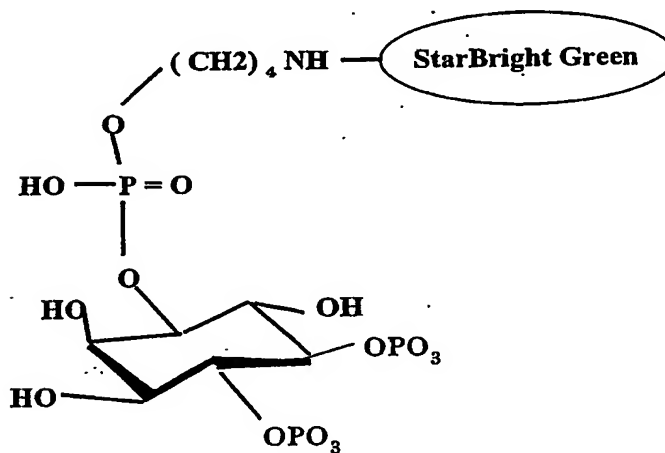


Figure 2.b. Water soluble lipid kinase target substrates: above, an example of the water soluble, StarBright-labeled derivatives of phosphatidyl inositol and its phosphorylated products. Alternative target substrates may be the single fatty acyl chain 1-StarBright Green -*myo*-inositol -1 phosphate lithium salts shown below and described in the text.

Arg - Phe - Ala - Arg - Lys - Gly - Ser - Leu - Arg - Gln - Lys - Asn - Val - COOH



Arg - Phe - Ala - Arg - Lys - Gly - Ser - Leu - Arg - Gln - Lys - Asn - Val - COOH

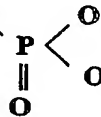


Figure 2. c. Peptide Target Substrate Phosphorylation -

The pseudosubstrate of Protein Kinase C-alpha and the site specific Phosphorylation of Serine by the PKC isozyme, PKC-theta

HO - CCA - ATC - TCA - TCT - TGT - TTT - CTG - CG - SPACER - StarBright Green

ATP, T4 nucleotide kinase, Ph 7.4

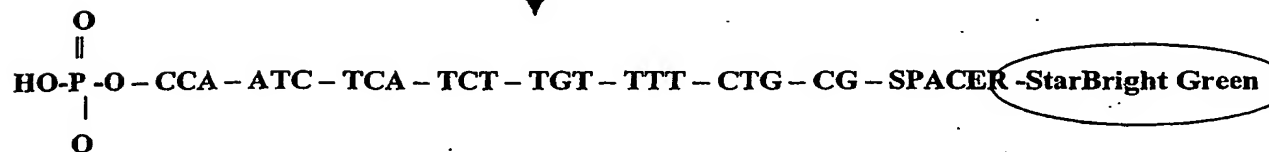
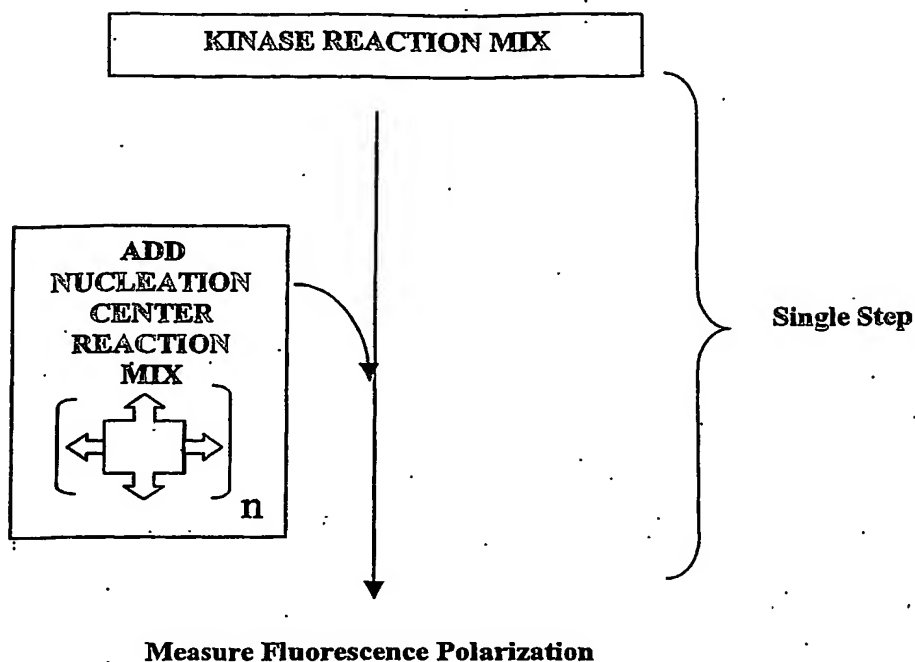


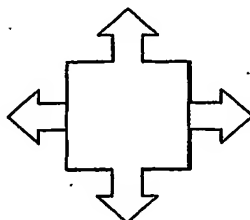
Figure 2.d. Oligonucleotide Target Substrate Phosphorylation -

The beta-actin target of T4 nucleotide kinase and the terminal phosphorylation of the oligonucleotide by the kinase

a) Single Step *Homogeneous* Assay using the rapid reaction method of the Present Invention

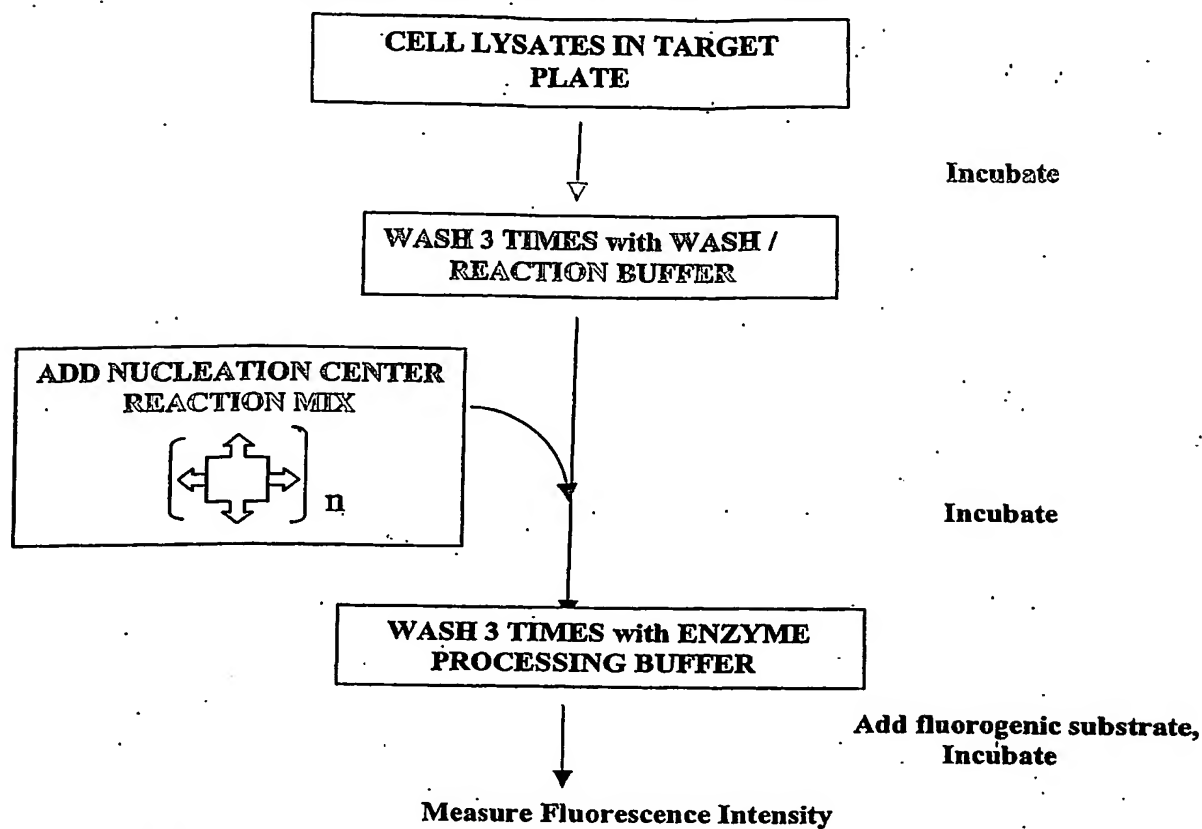


b) the "Nucleation Effect" in which multiple heterobifunctional linkers are attached to High Molecular weight core molecules such as avidin or another polymer to create a multi-valent reaction center that serves to enhance reaction rates,



where the square at the center represents the high molecular weight core that is conjugated to multiple copies ($n > 2$) of the heterobifunctional linkers (arrow heads) shown in Figure 1.

Figure 3. Schematic diagram (a) of the single step *homogeneous* assay method based upon the "nucleation effect" of the present invention and an idealized diagram (b) illustrating the nucleation effect itself;

a) Multi- Step *Heterogeneous* Assay of the Present Invention

b)

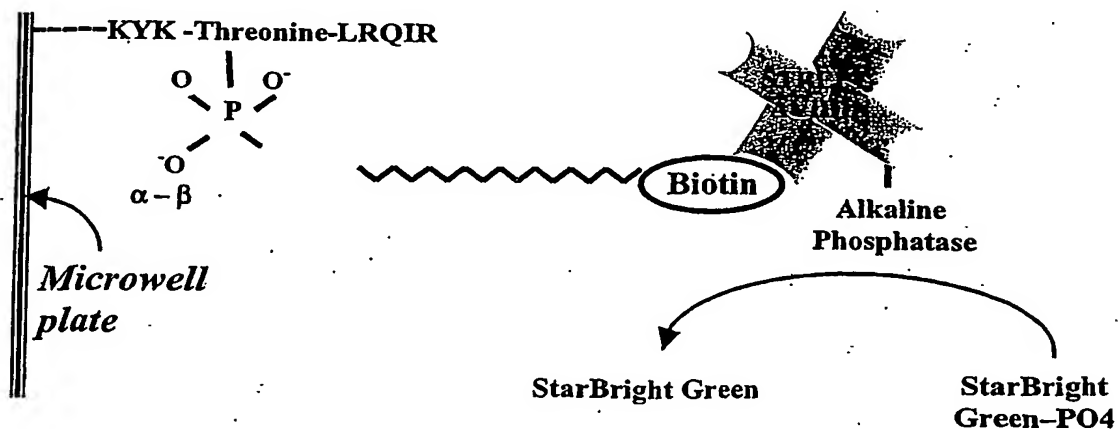


Figure 4. Schematic diagram (a) and mechanism (b) of the *heterogeneous* assay method : based upon the nucleation effect of the present invention

Phosphoramidate Chemistry For Developing Fluorescence Polarization Based Protein Kinase Assays

Schematic Representation of Steps Involved:

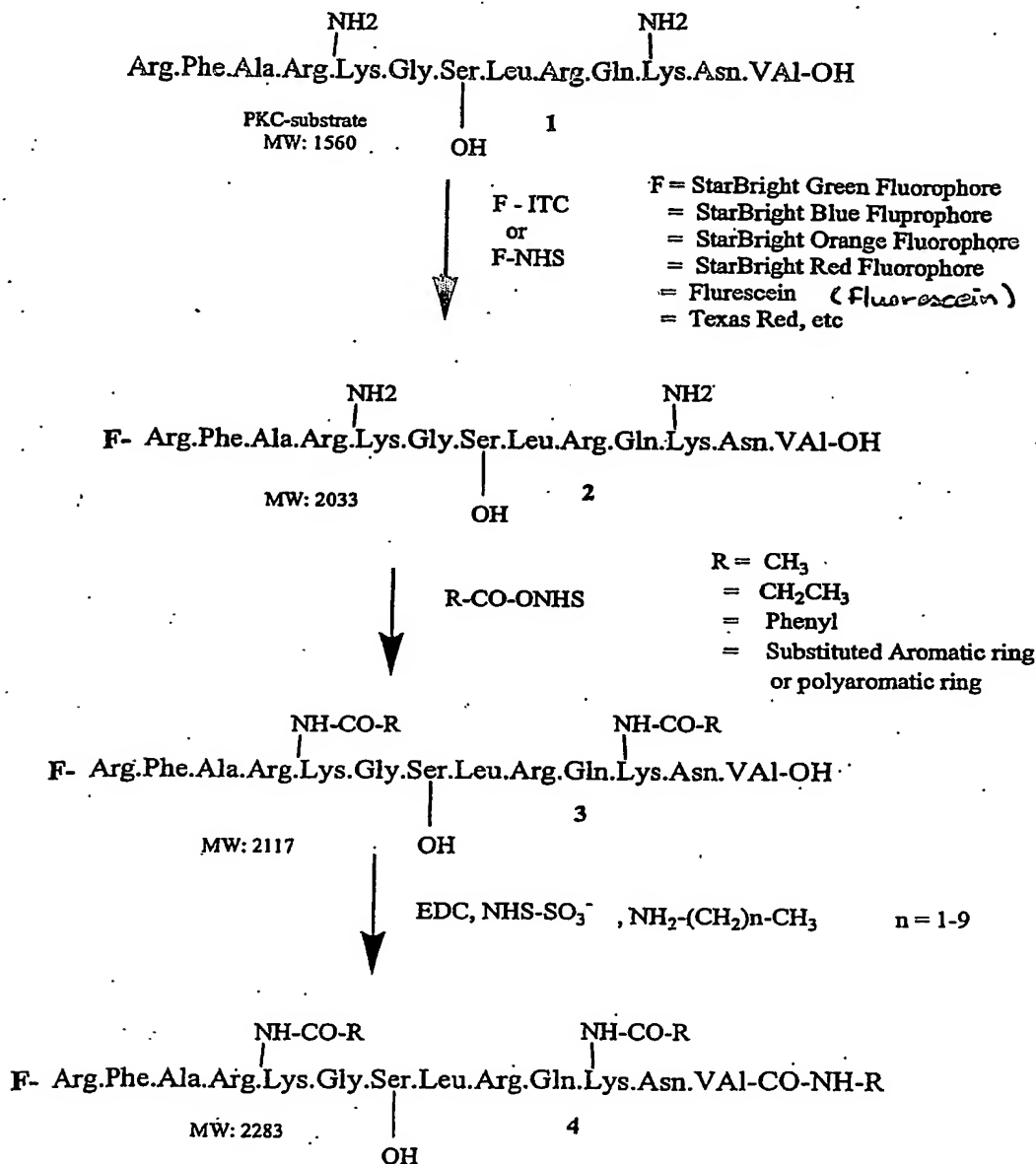


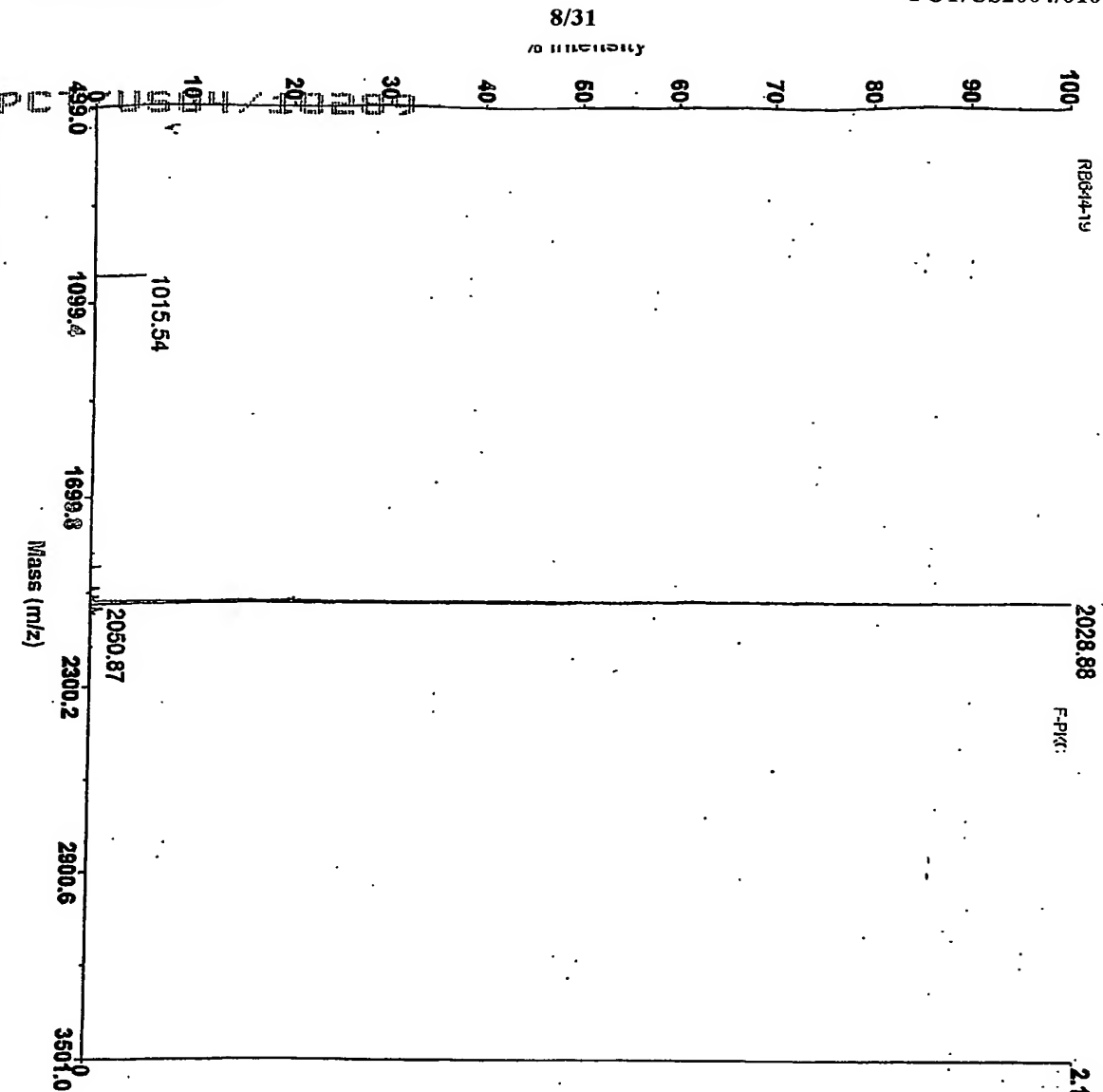
Figure 5. Novel protocols for blocking potentially reactive -NH₂ and -COOH groups on peptide targets of the present invention

Applied Biosystems Voyager System 1197

Voyager Spec #1[BP = 2028.9, 20777]

PCT/US2004/010289

WO 2004/089295



Mode of operation:
Extraction mode:
Polarity:
Acquisition control:

Linear
Delayed
Positive
Manual

2.1E+4 Accelerating voltage:

20000 V

Grid voltage:

95%

Guide wire D:

0.05%

Extraction delay time:

200 nsec

Acquisition mass range:

500 - 3800 Da

Number of laser shots:

100/spectrum

Laser intensity:

1548

Laser Rep Rate:

20.0 Hz

Calibration type:

Default

Calibration matrix:

8-Cyano-4-hydroxydynamic acid

Low mass gate:

500 Da

Digitizer start time:

14.258

Bin size:

2 nsec

Number of data points:

11636

Vertical scale:

1000 mV

Vertical offset:

0%

Input bandwidth:

150 MHz

Sample well:

01

Plate ID:

1

Serial number:

1197

Instrument name:

Voyager-DE

Plate type filename:

C:\VOYAGER\100 well plate.pit

Lab name:

PE Biosystems

Absolute x-position:

1398.02

Relative x-position:

47085.3

Relative y-position:

-189.476

Shots in spectrum:

212.193

Source pressure:

100

Mirror pressure:

1.178e-006

TC2 pressure:

0

TIS gate width:

0.0134

TIS flight length:

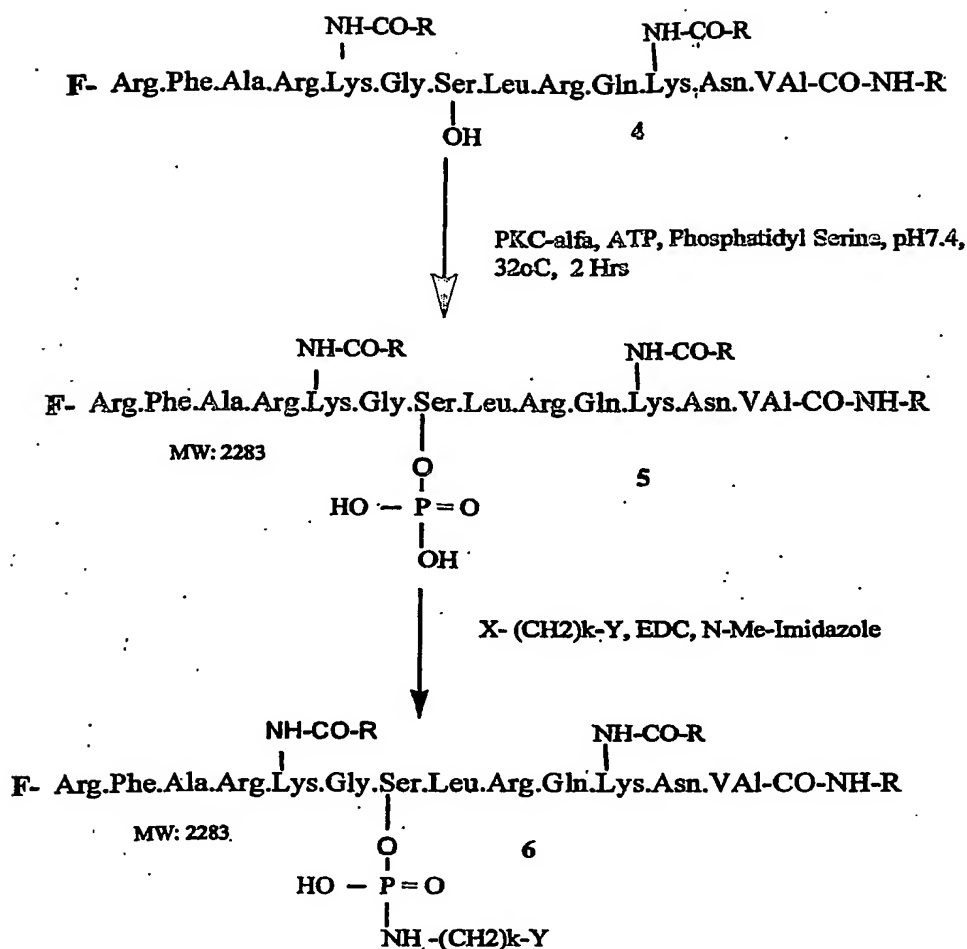
30

940

Figure 6.

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voyager\RAM\VB044-19\Frac3-7 August23, 02_0001.dcl

Maldi-Mass spectrum of the PKC-peptide target labeled with fluorescein at its N-terminal for the kinase activities of the isozymes of Protein Kinase C (PKC)



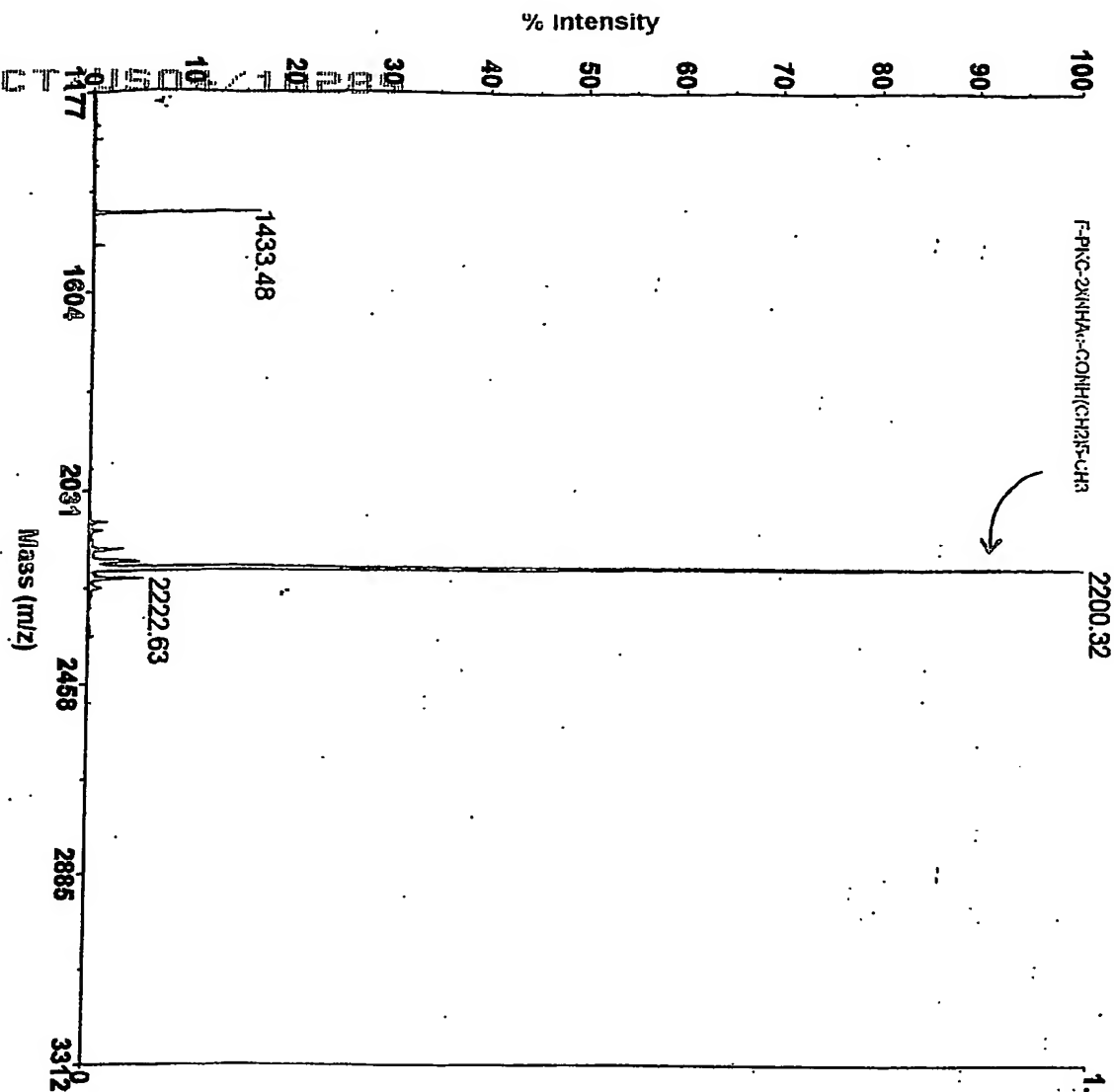
Where $k = 1-15$

and $Y =$ biotin, biotin-avidin complex, biotin-streptavidin complex, avidin-alkaline phosphatase (AP) conjugate, or unconjugated AP, b-Lactamase, Casein, or any other large molecular weight, including but not limited to antibodies, and derivatized particles.

Figure 7. Protocol and chemistry of the present invention for the formation of phosphor-amidates used in the detection of phosphoryl groups using the Nucleation Centers and rapid assay methods and phosphoramidate Chemistry I of the present invention

Applied Biosystems Voyager System 1197

Voyager Spec #11BP = 2199.9, 101171



Mode of operation: Linear
Extraction mode: Delayed
Polarity: Positive
Acquisition control: Manual

Accelerating voltage: 20000 V
Grid voltage: 95%
Guide wire O: 0.05%

Extraction delay time: 200 nsec

Acquisition mass range: 500 - 5000 Da
Number of laser shots: 100/spectrum

Laser intensity: 1605

Laser Rep Rate: 20.0 Hz

Calibration type: Default

Calibration matrix: a-Cyano-4-hydroxycinnamic acid

Low mass gate: 500 Da

Digitizer start time: 14.268

Bin size: 2 nsec

Number of data points: 16284

Vertical scale: 1000 mV

Vertical offset: 0%

Input bandwidth: 150 MHz

Sample well: 56

Plate ID: JEFF

Serial number: 1197

Instrument name: Voyager-DE

Plate type filename: C:\VOYAGER100 well plate.pit

Lab name: PE Biosystems

Absolute x-position: 26803.1

Absolute y-position: 21783.4

Relative x-position: -184.412

Relative y-position: -154.076

Sticks in spectrum: 100

Source pressure: 1.332e-006

Micro pressure: 0

TC2 pressure: 0.01227

TIS gate width: 30

TIS flight length: 940

Figure 8a: MALDI-MS of fully protected fluoresceinated PKC peptide target that potential reactive sites blocked as described in figure 5

27, March 10, 2003

11/31

Applied Biosystems Voyager System 1197
Voyager Spec #11BP = 2279.6, 38141

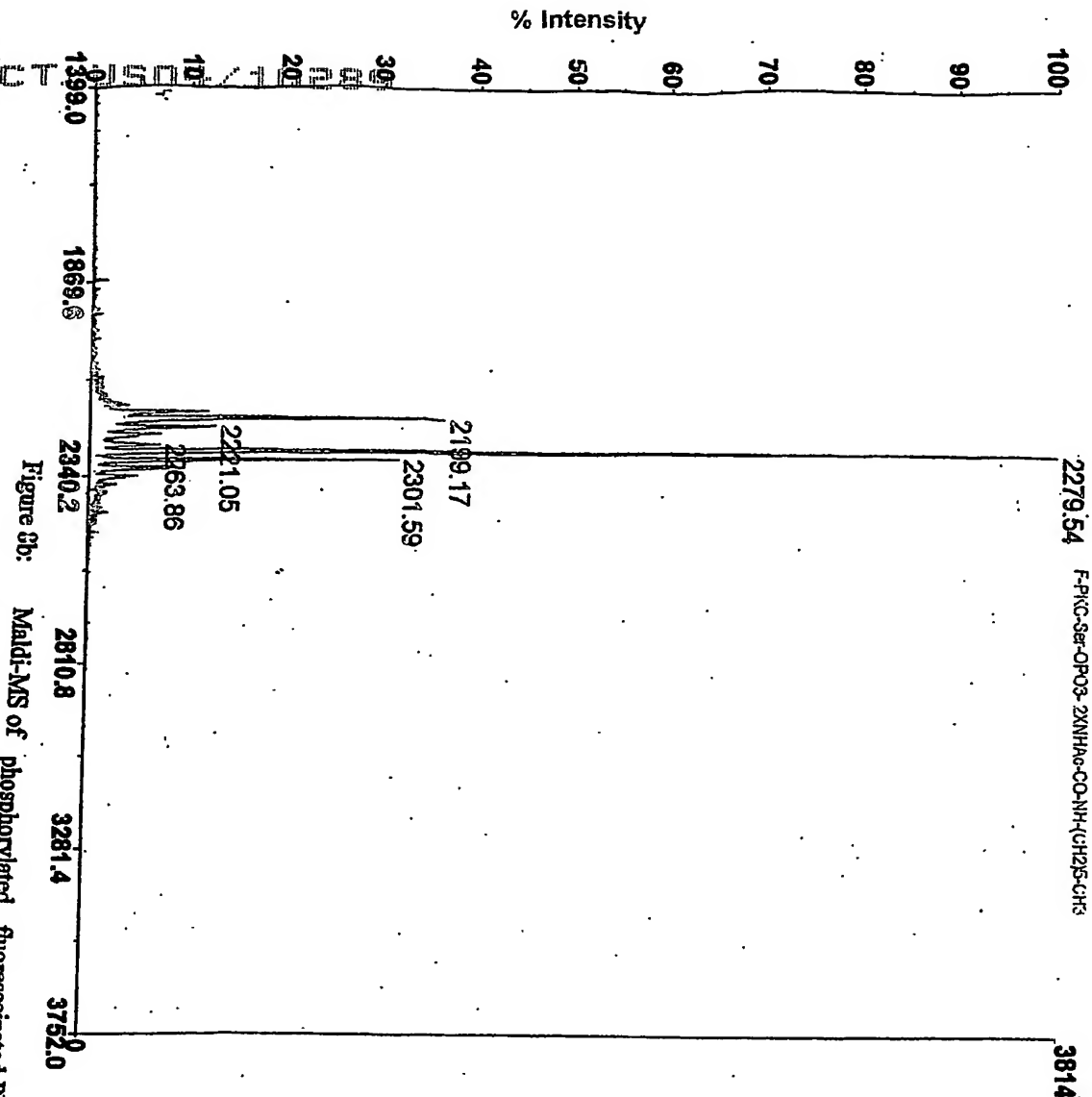


Figure 3b:

Maldi-MS of phosphorylated, fluoresceinated-PKC peptide target that had potential sites blocked as described in Example 1 *before* the addition of multiplexed Nucleation Centers that had been performed from avidin and the heterobifunctional biotin linkers of chemistry 1.

Mode of operation:	Linear
Extraction mode:	Delayed
Polarity:	Positive
Acquisition control:	Manual
Accelerating voltage:	20000 V
Grid voltage:	95%
Guide wire O:	0.05%
Extraction delay time:	200 nsec
Acquisition mass range:	500 - 5000 Da
Number of laser shots:	100/spectrum
Laser intensity:	1640
Laser Rep Rate:	20.0 Hz
Calibration type:	Default
Low mass gate:	a-Cyano-4-hydroxycinnamic acid
Digitizer start time:	14.258
Bin size:	2 nsec
Number of data points:	16284
Vertical scale:	1000 mV
Vertical offset:	0%
Input bandwidth:	150 MHz
Sample well:	55
Plate ID:	JEFF
Serial number:	1197
Instrument name:	Voyager-DE
Plate type filename:	C:\VOYAGER\100 well plate.pl
Lab name:	PE Biosystems
Absolute x-position:	21178.3
Absolute y-position:	20890.3
Relative x-position:	-728.201
Relative y-position:	-1017.22
Shots in spectrum:	100
Source pressure:	6.258e-007
Minor pressure:	0
TC2 pressure:	0.00844
TIS gate width:	30
TIS flight length:	940

Applied Biosystems Voyager System 1197

Voyager Spec #1 [BP = 1762.0, 13304]

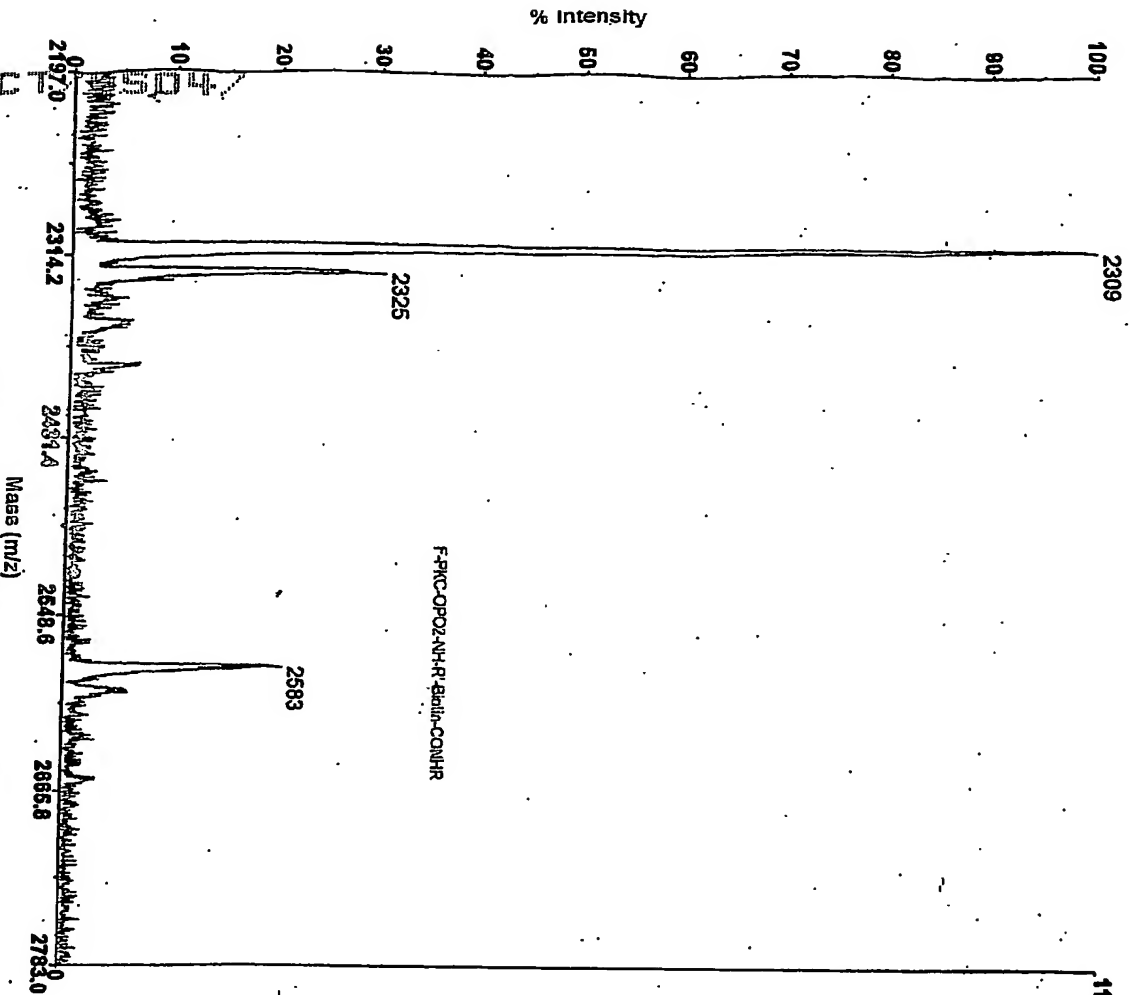


Figure 8c:

Mass (m/z)

Acquired: 17/3/2003, November 19, 2001
 VoyagerRAMSBI0011001_0001.dad

Multi-MS of phosphoramidated, fluoresceinated PKC peptide target that had potential reactive sites blocked before the addition of multiplexed preformed Nucleation Centers from avidin and heterobifunctional biotin

Printed: 18:03, March 10, 2003

Mode of operation: Linear
 Extraction mode: Delayed
 Polarity: Positive
 Acquisition control: Manual

1162.0 Accelerating voltage: 20000 V
 Grid voltage: 95%
 Guide wire On: 0.05%
 Extraction delay time: 200 nsec

Acquisition mass range: 1500 - 3000 Da
 Number of laser shots: 1000/spectrum
 Laser intensity: 1981
 Laser Rep Rate: 20.0 Hz
 Calibration type: External - DiVoyagerData/cal2 nov13_0001.cal
 Calibration method: e-Cyano-4-hydroxycinnamic acid
 Low mass gate: 1500 Da

Digitizer start time: 24.69
 Bin size: 2 nsec
 Number of data points: 5089
 Vertical scale: 1000 mV
 Vertical offset: 0%
 Input bandwidth: 150 MHz

Sample well: 01
 Plate ID: 1
 Serial number: 1197
 Instrument name: Voyager-DE
 Plate type filename: C:\VOYAGER\100 well plate.plt
 Lab name: PE Biosystems

Absolute X-position: 1883.1
 Absolute Y-position: 47312.8
 Relative X-position: 285.604
 Relative Y-position: 6.30846
 Shots in spectrum: 100
 Source pressure: 4.304e-007
 Mirror pressure: 0
 TC2 pressure: 0.00875
 TIS gate width: 30
 TIS flight length: 840

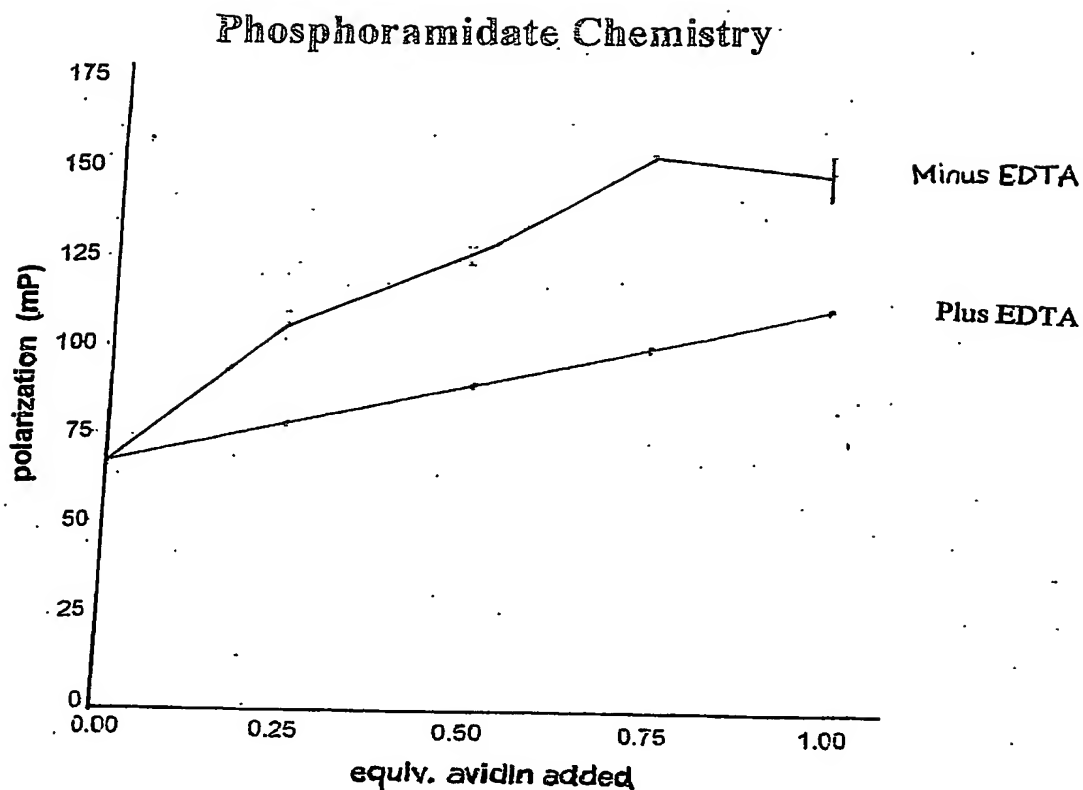


Figure 9. Fluorescence Polarization analysis of the stoichiometry of Nucleation Center Binding. The phosphoramidated PKC peptide target shown in Figure 7 after the addition of varying amounts of multiplexed Nucleation Centers using the linkers of Chemistry I. The two samples differed in that the negative controls were performed in the presence of 5mMolar EDTA which destroys the activity of the kinase.

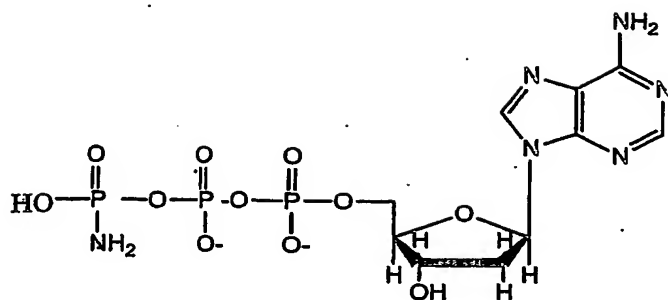
Structure of γ -NH₂-ATP:**7**

Figure 10. Chemical structure of the ATP structural analog, γ -Amino ATP (γ -NH₂-ATP)

Synthesis of γ -Amino-ATP

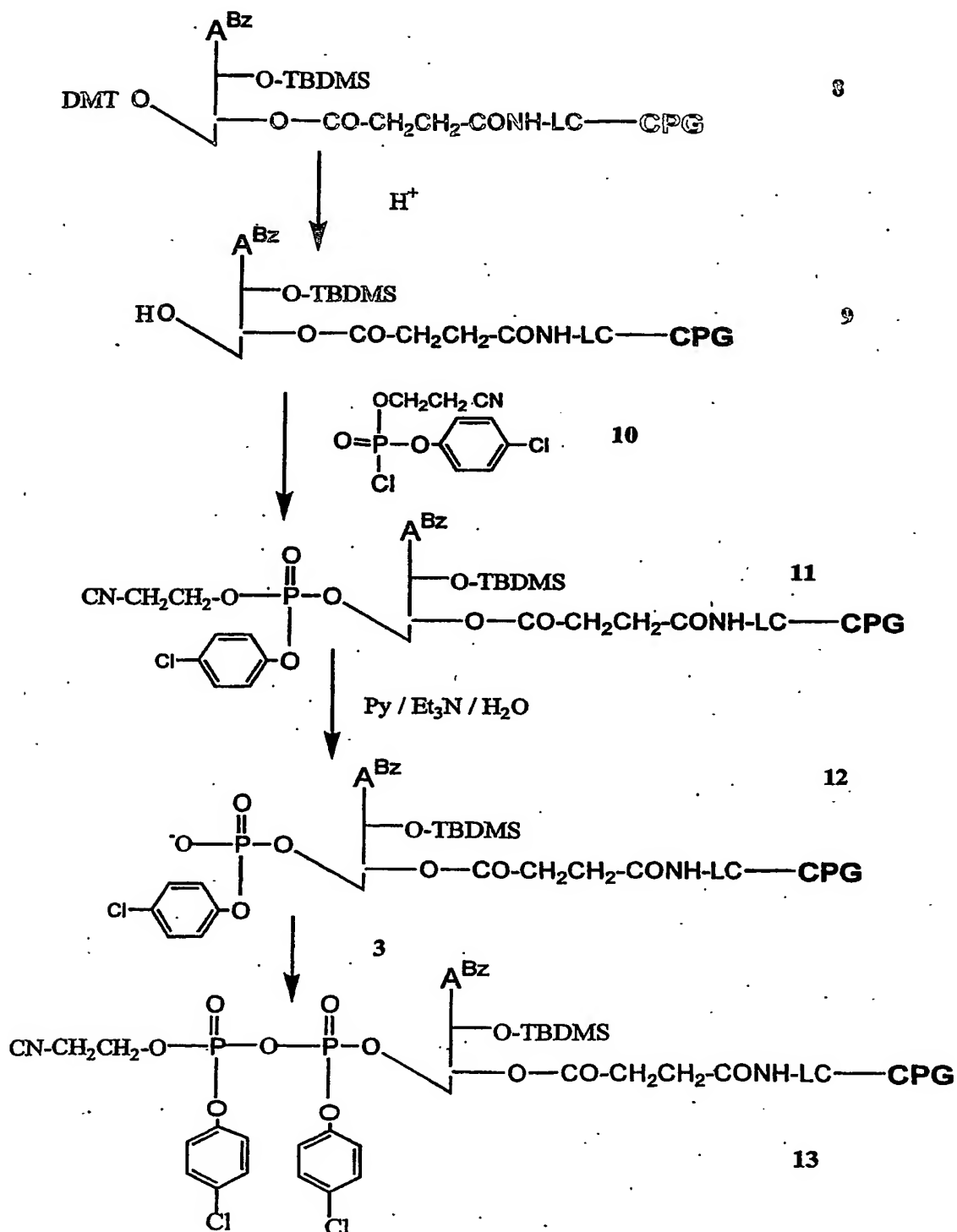


Figure 11. Protocol and chemistry of the present invention for the synthesis of γ -NH₂-ATP

Scheme for the synthesis of gamma-Amino-ATP continues

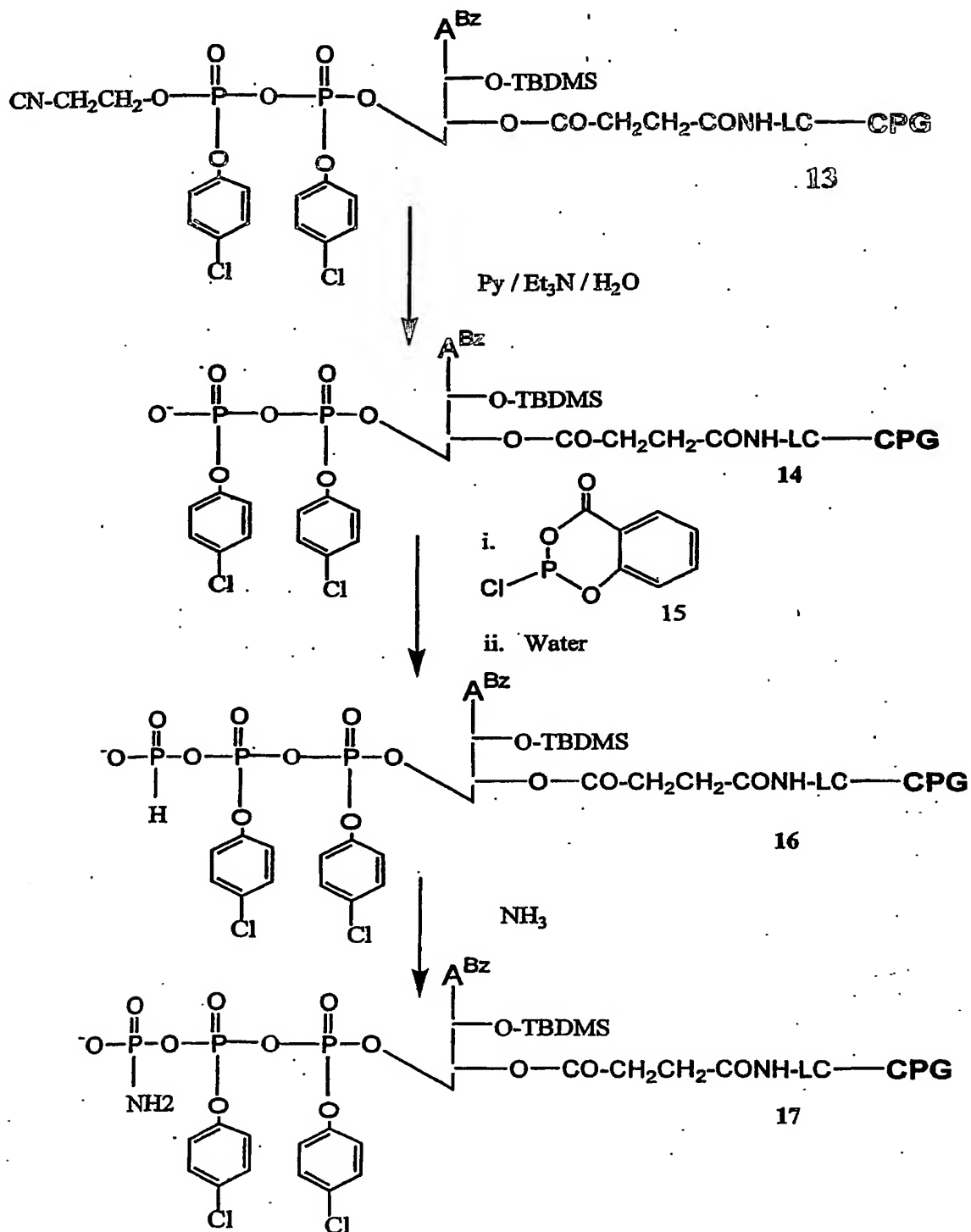
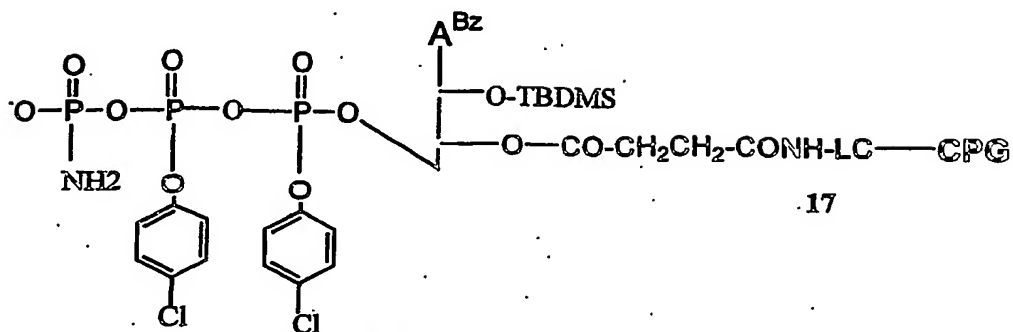
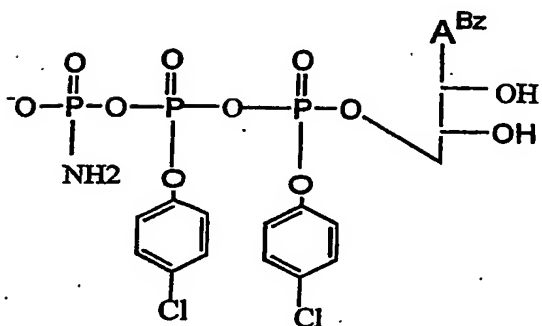


Figure 11: continuation (page 2)

Page 3 of Synthesis of γ -Amino-NH₂



- i. TMG, RT, 4 Hours
ii. NH₄OH, 60°C, 8 hours
iii. Concentrate to dryness under vacuum
iv. TBAF, RT, 16 hours



γ -Amino-NH₂

Figure 11: continuation (page 3)

Alternative Approach For Monitoring the Activity of Protein Kinases.

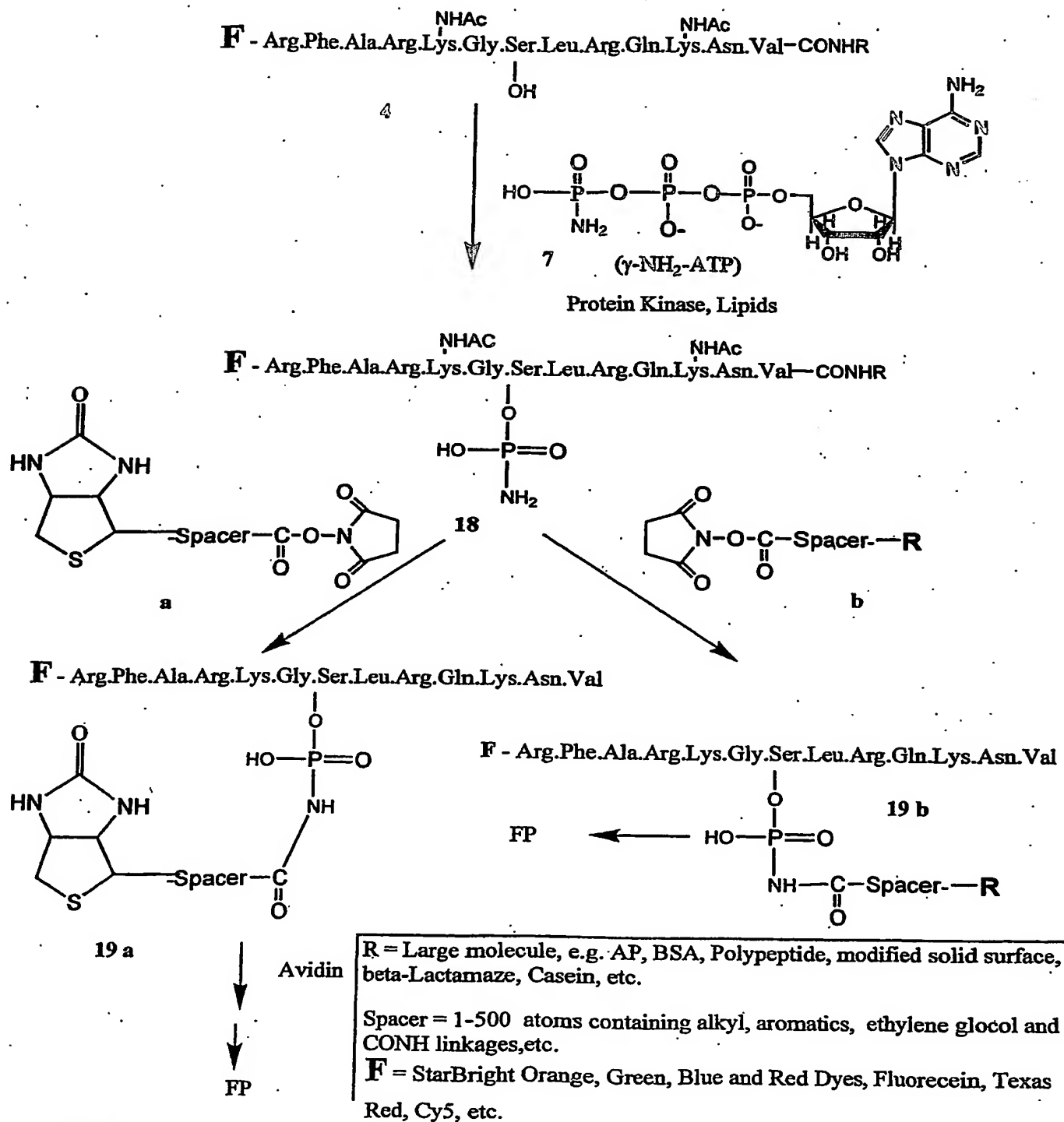
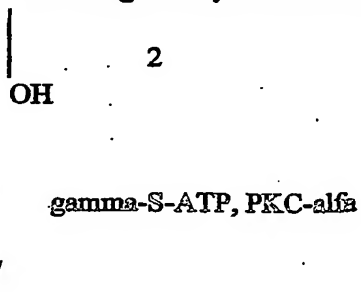


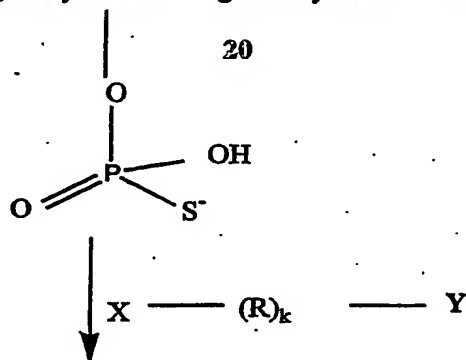
Figure 12. Procedure for phosphoroamidation of fluoresceinated -PKC peptide target using PKC-alpha and $\gamma\text{-NH}_2\text{-ATP}$

Phosphorothioate Chemistry

F- Arg.Phe.Ala.Arg.Lys.Gly.Ser.Leu.Arg.Gln.Lys.Asn.Val-OH



F- Arg.Phe.Ala.Arg.Lys.Gly.Ser.Leu.Arg.Gln.Lys.Asn.Val-OH

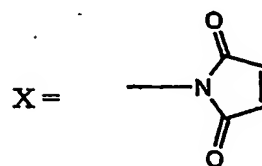
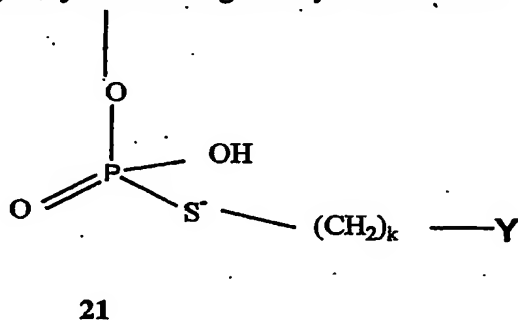


where k= 1-100

R= Alkyl, alkoxyl, cycloalkanyl,
aromatic, heterocyclic,
ethylene glycolic, peptidyl, etc

Y= Biotin, Biotin-Avidin,
Biotin-Streptavidin, or Large
Polymer such as Alkaline
Phosphatase (AP), Streptavidin
(SA), Casein, glycoprotein, IgG,
enzyme, DNA, RNA with or
without conjugation to Avidin

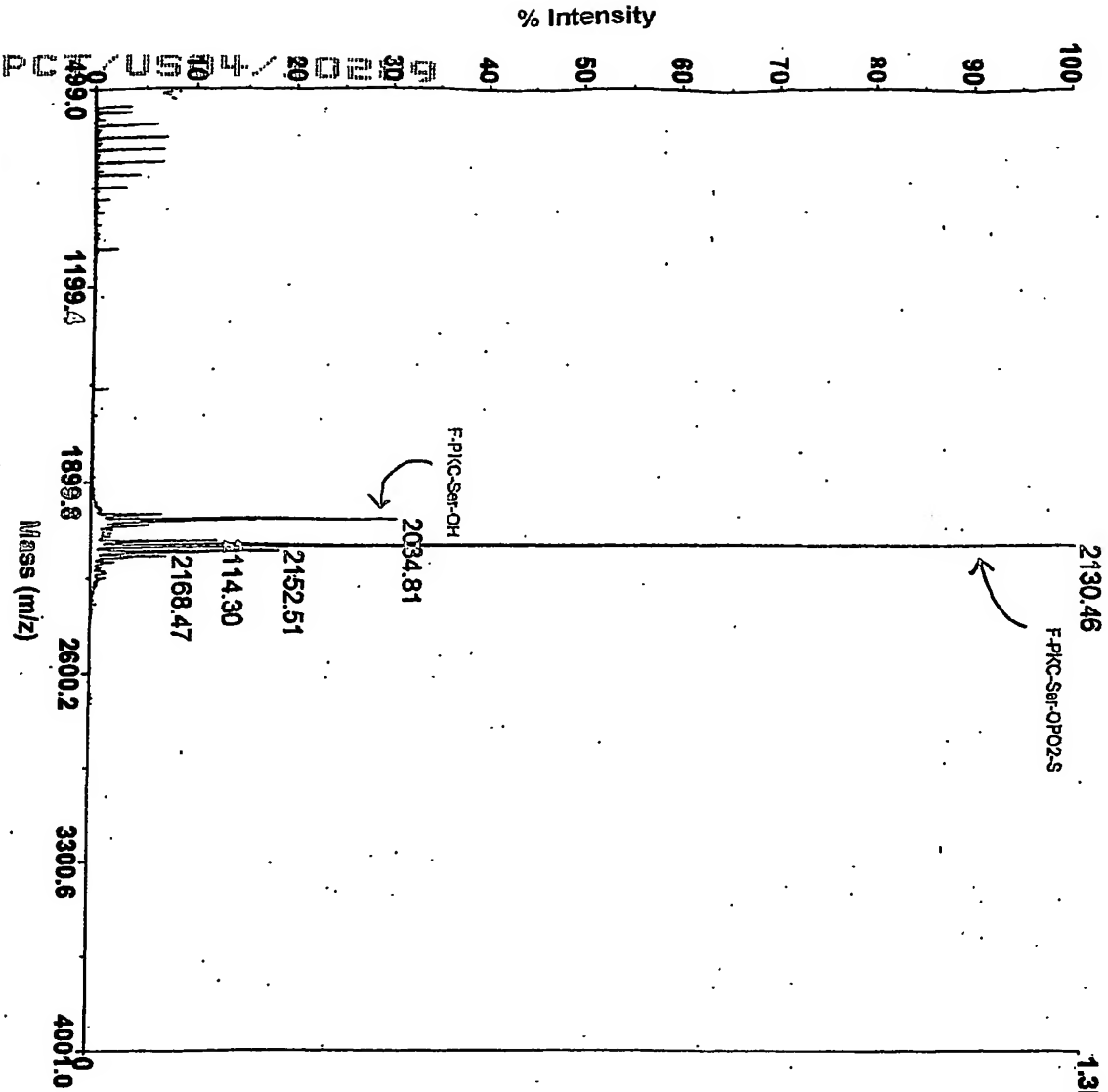
F- Arg.Phe.Ala.Arg.Lys.Gly.Ser.Leu.Arg.Gln.Lys.Asn.Val-OH



X = I-CH₂CONH--
= Br-CH₂CONH--
= S

Figure 13. Protocol and chemistry of the present invention for phosphorothiolation and detection of fluoresceinated PKC-peptide target using the single step, nucleation effect rapid assay method and Chemistry III of the present invention

Applied Biosystems Voyager System 1197
Voyager Spec #1[BP = 2130.5, 13023]



Mode of operation: Linear
 Extraction mode: Delayed
 Polarity: Positive
 Acquisition control: Manual

Accelerating voltage: 20000 V
 Grid voltage: 95%
 Guide wire Q: 0.05%
 Extraction delay time: 200 msec

Acquisition mass range: 600 -- 4000 Da
 Number of laser shots: 100/spectrum
 Laser intensity: 1284
 Laser Rep Rate: 20.0 Hz
 Calibration type: Default
 Calibration matrix: s-Cyano-L-hydroxycinnamic acid
 Low mass gate: 500 Da

Digitizer start time: 14.298
 Bin size: 2 msec
 Number of data points: 12860
 Vertical scale: 1000 mV
 Vertical offset: 0%
 Input bandwidth: 150 MHz

Sample well: 23
 Plate ID: PLATE1
 Serial number: 1197
 Instrument name: Voyager-DE
 Plate type filename: C:\VOYAGER\100 well plate.plt
 Lab name: PE Biosystems

Abscissa x-position: 11746.9
 Abscissa y-position: 37147.9
 Relative x-position: -0.690595
 Relative y-position: 0.398375
 Shots in spectrum: 100
 Source pressure: 7.869e-007
 Mirror pressure: 0
 TCD pressure: 0.01229
 TIS gate width: 30
 TIS flight length: 940

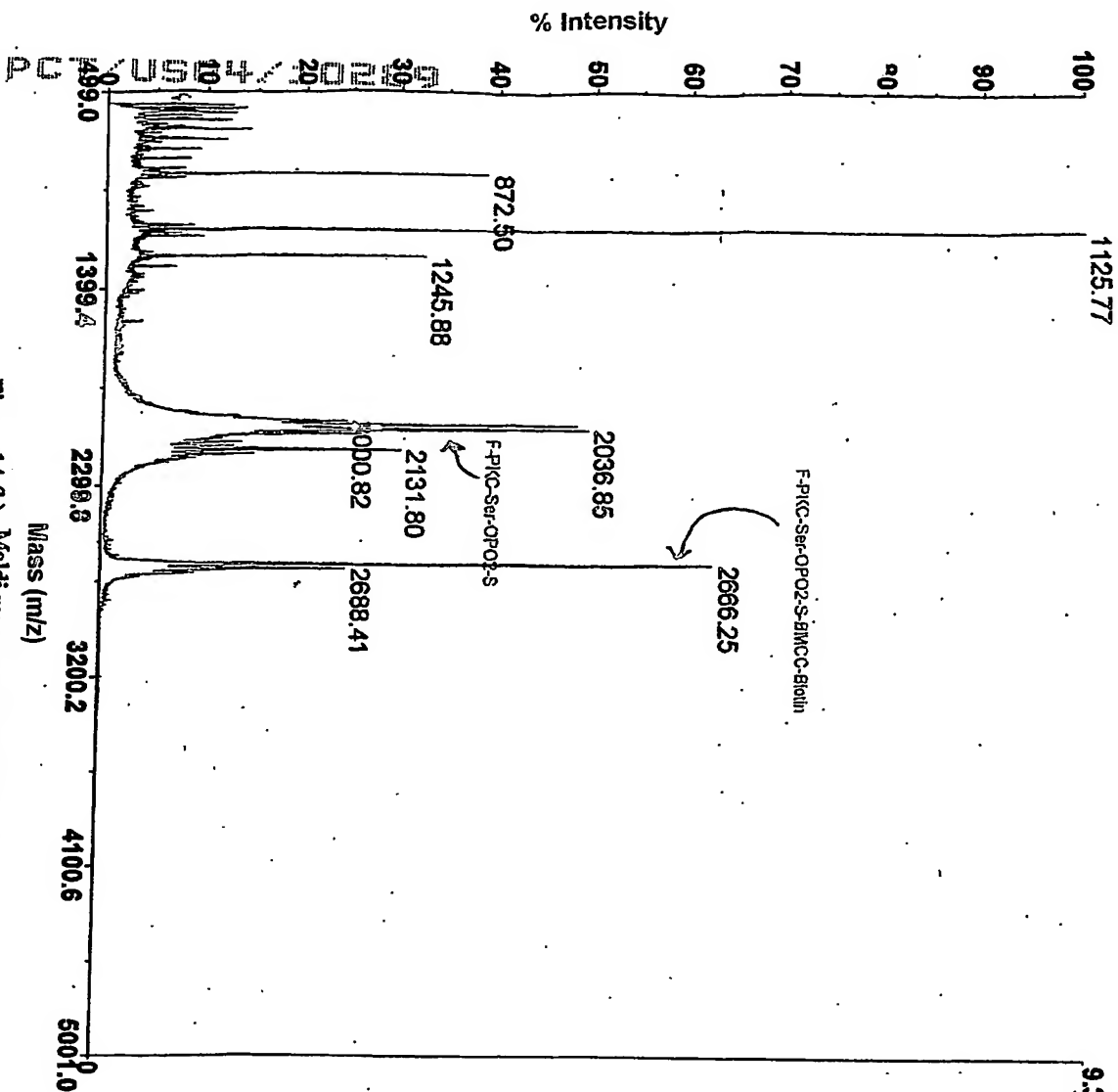
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 File: \vsc\data\18889-1180\132_0001.dat

Figure 14 (a): Maldi-MS of Phosphorothiolated fluoresceinated -PKC peptide target

Printed: 16:24, March 11, 2003

Applied Biosystems Voyager System 1197

Voyager Spec #11BP = 1125.6, 9311]



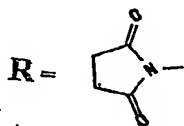
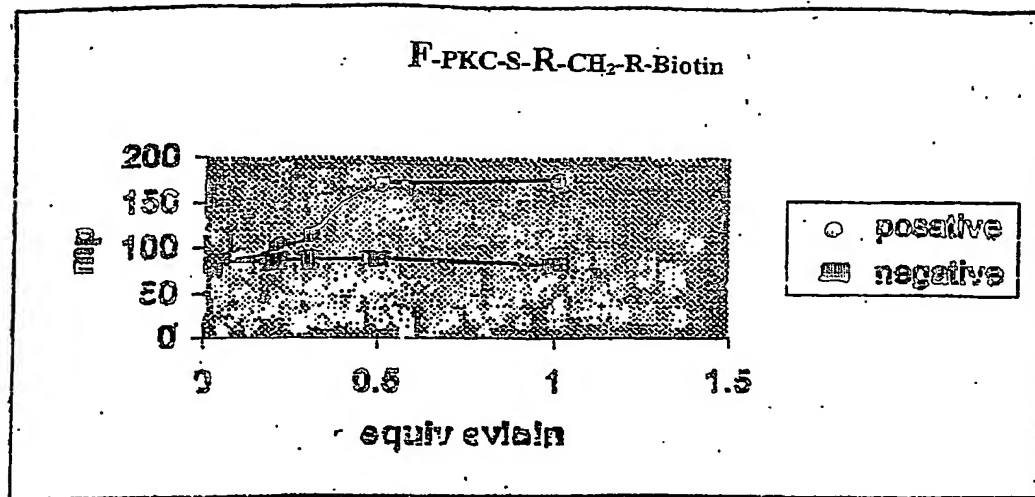
Acquired: 17/10/00, September 21, 2001

\\voyager\data\RB558-11-B1_0002.dct

Figure 14 (b):

Maldi mass spectrum of the same phosphorothiolated, fluoresceinated PKC peptide target 15 minutes after addition of equimolar equivalents multiplexed Nucleon Centers preformed from avidin and the hetero-bifunctional biotin.

Mode of operation:	Linear
Extraction mode:	Delayed
Polarity:	Positive
Acquisition control:	Manual
Accelerating voltage:	20000 V
Grid voltage:	95%
Guide wire on:	0.05%
Extraction delay time:	200 nsec
Acquisition mass range:	500 - 5000 Da
Number of laser shots:	100/spectrum
Laser intensity:	1640
Laser Rep Rate:	20.0 Hz
Calibration type:	Default
Calibration matrix:	s-Cyano-4-hydroxycinnamic acid
Low mass gate:	500 Da
Digitizer start time:	14.268
Bin size:	2 nsec
Number of data points:	15324
Vertical scale:	1000 mV
Vertical offset:	0%
Input bandwidth:	150 MHz
Sample well:	47
Plate ID:	RB109FEB21
Serial number:	1197
Instrument name:	Voyager-DE
Plate type filename:	C:\VOYAGER\100 well plate.pil
Lab name:	PE Biosystems
Absolute x-position:	32051.3
Absolute y-position:	26104.1
Relative x-position:	-16.1985
Relative y-position:	-883.367
Shot in spectrum:	100
Source pressure:	9.673e-007
Mirror pressure:	0
TC2 pressure:	0.01058
TIS gate width:	30
TIS flight length:	940



$R = \text{Long Chain alkyl}$

Figure 14 (C) Fluorescence polarization analysis of the same sample used to generate the spectrum of 14(b), above, showing the titration with multiplexed Nucleation Centers that were prepared from avidin and the hetero-bifunctional linkers, maleimido BMCC-biotin.

Applied Biosystems Voyager System 4197

Voyager Spec #1[BP = 2135.2, 10132]

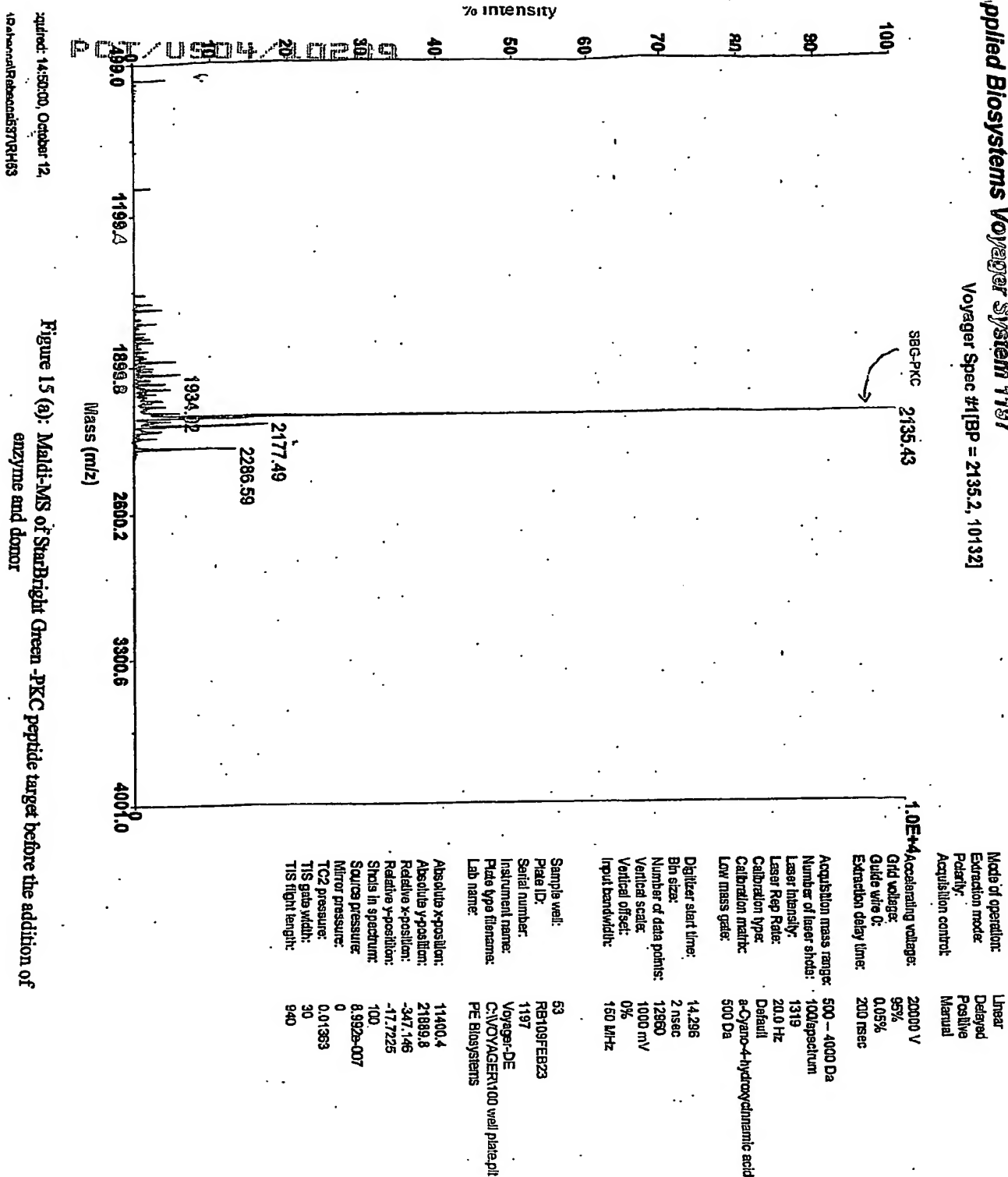
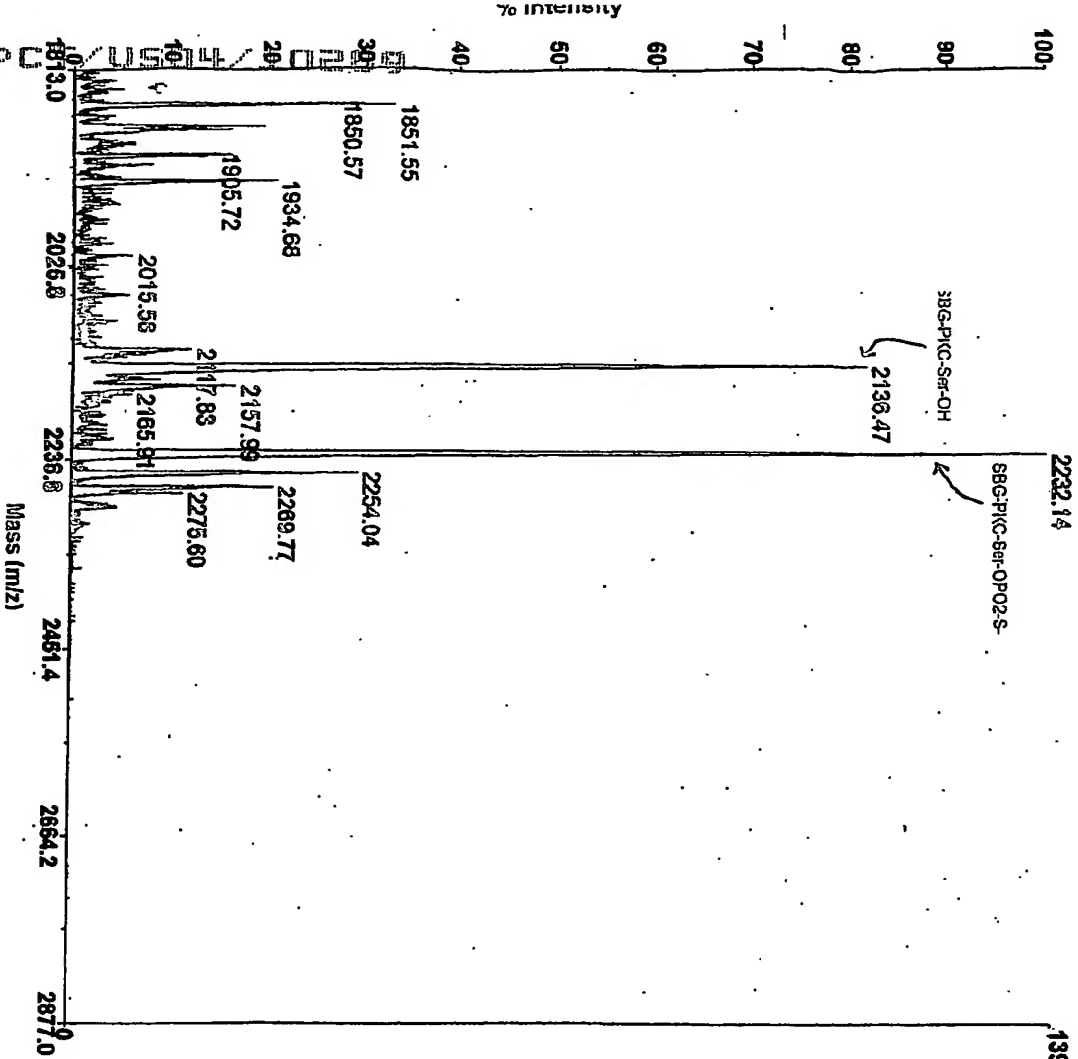


Figure 15 (a): Maldi-MS of StarBright Green -PKC peptide target before the addition of enzyme and donor

ADOC 37B71VAV L2SB

Applied Biosystems Voyager System 1107

Voyager Spec #11BP = 2231.9, 13981



Mode of operation: Linear
Extraction mode: Delayed
Polarity: Positive
Acquisition control: Manual

Accelerating voltage: 20000 V
Grid voltage: 95%
Guide wire V: 0.05%
Extraction delay time: 200 nsec

Acquisition mass range: 1000 - 4000 Da
Number of laser shots: 1000/spectrum
Laser intensity: 1319
Laser Rep. Rate: 20.0 Hz
Calibration type: Default
Calibration matrix: e-Cyano-4-hydroxycinnamic acid
Low mass gate: 1000 Da

Diluter alert time: 20.17
BIN size: 2 nsec
Number of data points: 10023
Vertical scale: 1000 mV
Vertical offset: 0%
Input bandwidth: 150 MHz

Sample well: 24
Plate ID: RB109FEB23
Serial number: 1197
Instrument name: Voyager-DE
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: PE Biosystems

Absolute X-position: 16216.2
Absolute Y-position: 37102.9
Relative X-position: -611.266
Relative Y-position: -44.6175
Shots in spectrum: 100
Source pressure: 1.34e-006
Mirror pressure: 0
TIC2 pressure: 0.01205
TIS gate width: 30
TIS flight length: 940

Figure 15 (b): MALDI-MS of Phosphorothiolated StarBright Green -PKC peptide target with enzyme and γ-S-ATP

Applied Biosystems Voyager System 1107

F-PKC-Voyager Spec #11BP = 1694.6, 104831

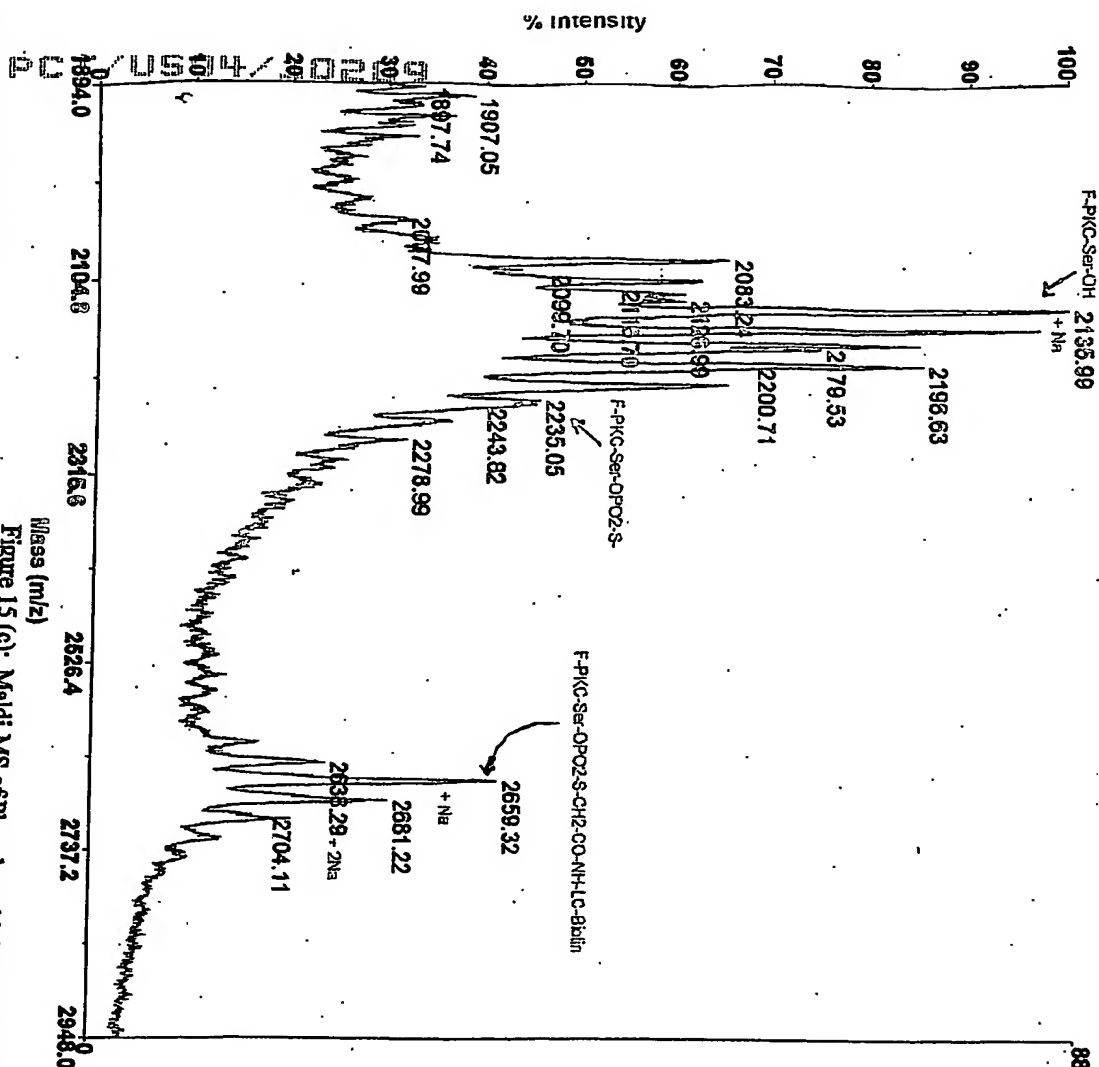


Figure 1.5 (c):

Maldi-MS of Phosphorothiolated StarBright Green -PKC peptide target
After the addition of multiplexed Nucleation Centers performed from
Biotin and the maleimido-heterobifunctional linker.

Mode of operation: Linear
Extraction mode: Delayed
Polarity: Positive
Acquisition control: Manual

8817.2 Accelerating voltage: 20000 V
Grid voltage: 95%
Guide wire O: 0.05%
Extraction delay time: 200 nsec

Acquisition mass range: 1000 - 3500 Da
Number of laser shots: 100/spectrum
Laser intensity: 1747
Laser Rep Rate: 20.0 Hz
Calibration type: Default
Calibration matrix: e-Cyano-4-hydroxycinnamic acid
Low mass gate: 1000 Da

Digitizer scan time: 20.17
Bin size: 2 nsec
Number of data points: 8728
Vertical scale: 1000 mV
Vertical offset: 0%
Input bandwidth: 150 MHz

Sample well: 24
Plate ID: RB109FEB23
Serial number: 1197
Instrument name: Voyager-DE
Plate type filename: C:\VOYAGER\100 well plate.plt
Lab name: PE Biosystems

Absolute x-position: 17820.6
Absolute y-position: 37815
Relative x-position: 793.118
Relative y-position: 687.548
Shots in spectrum: 100
Source pressure: 1.316e-008
Mirror pressure: 0
TC2 pressure: 0.0114
TIS gate width: 30
TIS flight length: 940

Fluorescence Polarization

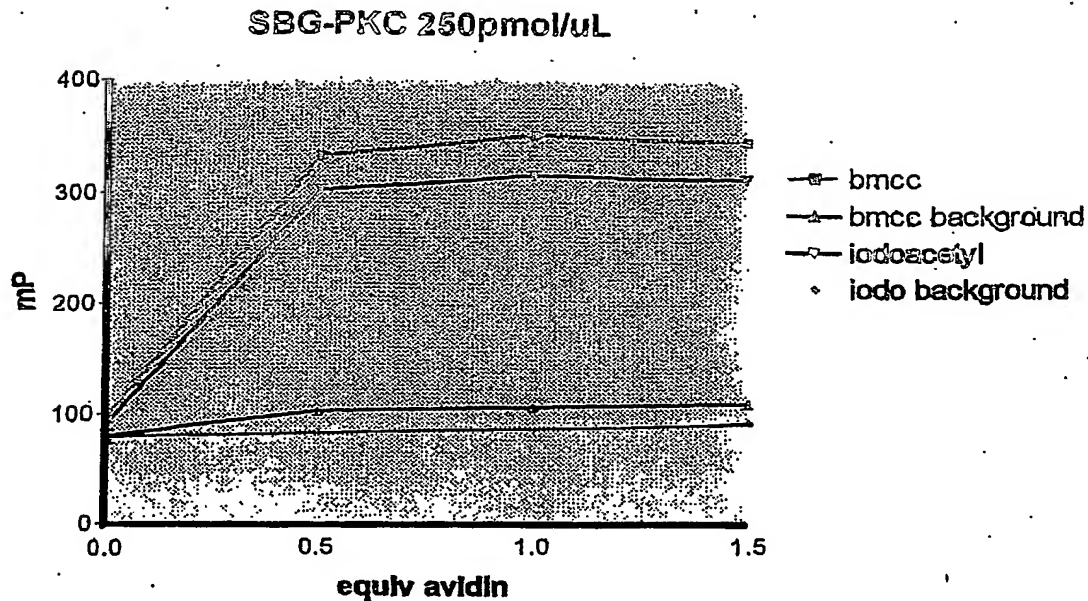
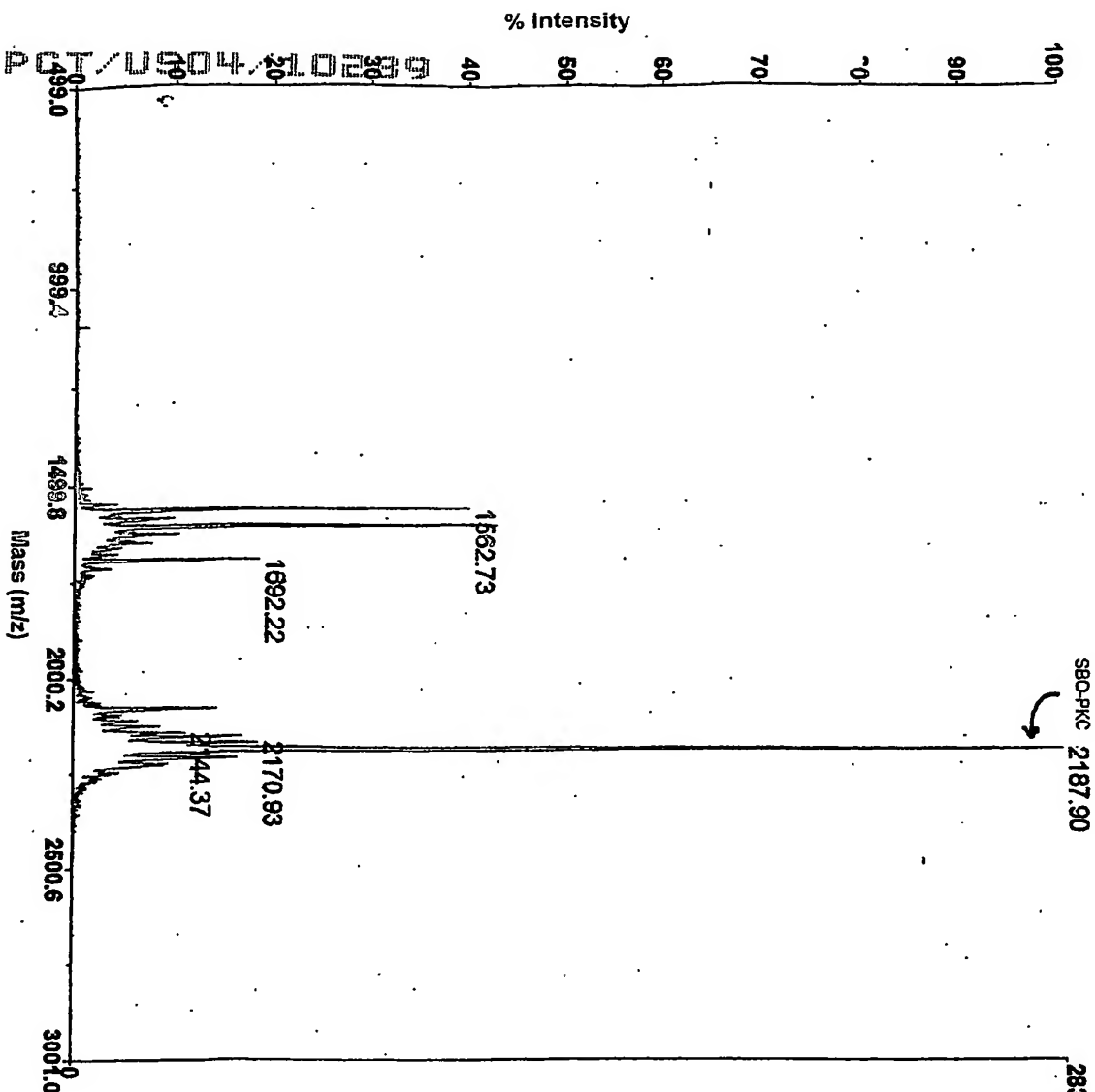


Figure 16. Fluorescence Polarization Analysis of the extent of the reaction of multiplexed Nucleation Centers prefomed from avidin and multiple heterobifunctional linkers bearing biotin at one terminus and maleimido- (blue line) and iodoacetamido- reactive groups at the other (purple line). The StarBright Green -PKC peptide target was phosphorylated by PKC-theta using γ -S-ATP as the donor.

Applied Biosystems Voyager System 1197

Voyager Spec #1 [BP = 2187.1, 2839]



Mode of operation: Linear
 Extraction mode: Delayed
 Polarity: Positive
 Acquisition control: Manual

Accelerating voltage: 20000 V
 Grid voltage: 85%
 Guide wire C: 0.05%
 Extraction delay time: 200 nsec

Acquisition mass range: 500 - 3000 Da
 Number of laser shots: 100/spectrum
 Laser intensity: 1141
 Laser Rep Rate: 20.0 Hz
 Calibration type: Default
 Calibration matrix: e-Cyano-4-hydroxycinnamic acid
 Low mass gate: 500 Da

Digitizer start time: 14.296
 Bin size: 2 nsec
 Number of data points: 10276
 Vertical scale: 1000 mV
 Vertical offset: 0%
 Input bandwidth: 150 MHz

Sample well: 65
 Plate ID: RB109FEB21
 Serial number: 1197
 Instrument name: Voyager-DE
 Plate type filename: C:\VOYAGER\1100 well plate.pit
 Lab name: PE Biosystems

Absolute x-position: 22254
 Absolute y-position: 16616.6
 Relative x-position: 346.452
 Relative y-position: -210.939
 Shots in spectrum: 100
 Source pressure: 8.339e-007
 Mirror pressure: 0
 TCO pressure: 0.01124
 TIS gate width: 30
 TIS flight length: 940

acquired: 16:44:00, August 22, 2001

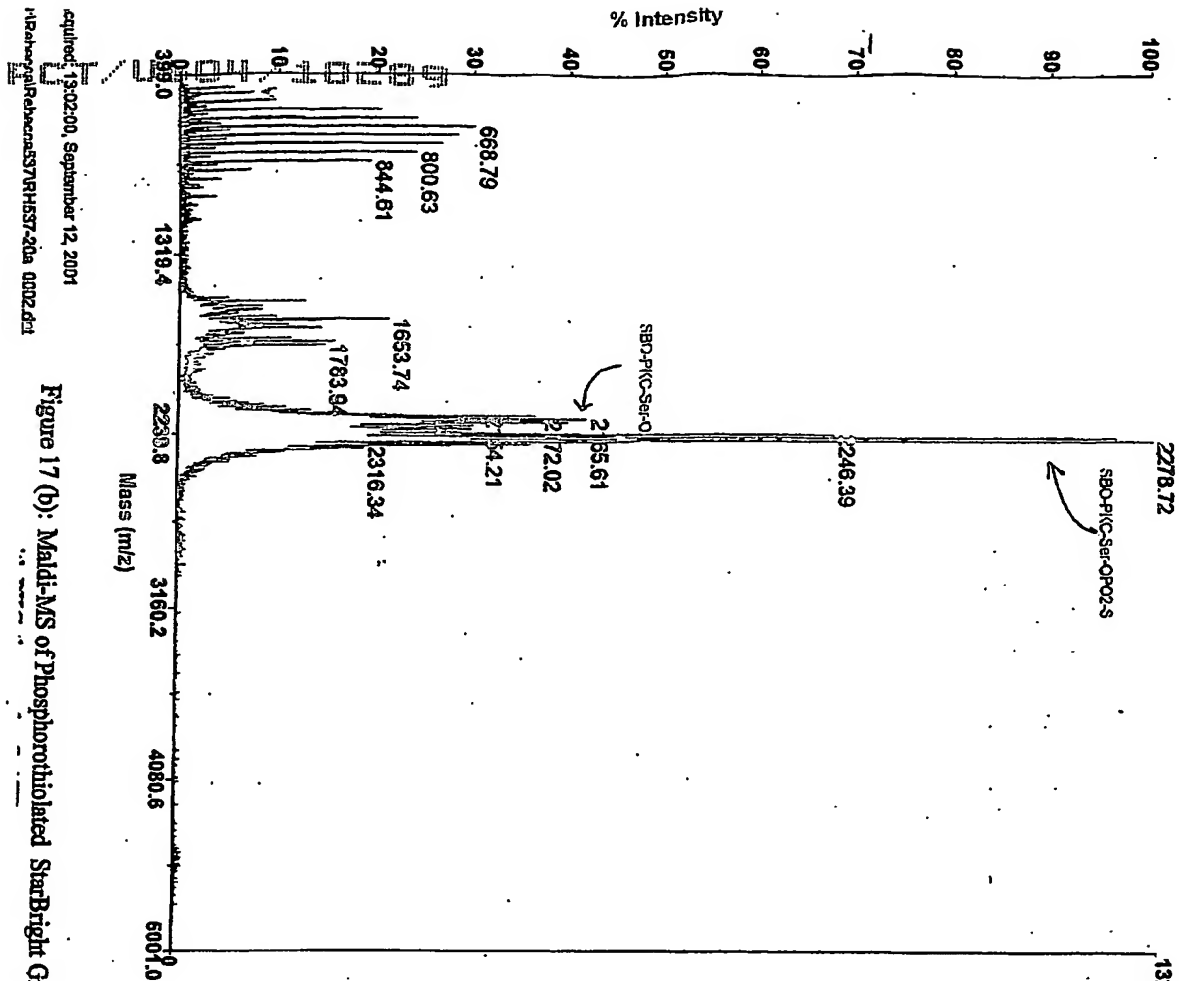
*D:\sharma\Biosystems\537\RI-E37-1.d\acq1

Figure 17 (a): MALDI-MS of StarBright Orange -PKC peptide target before the addition of enzyme and donor

arch 12, 2003

Applied Biosystems Voyager System 1197

Voyager Spec #1 [BP = 2277.8, 1326]

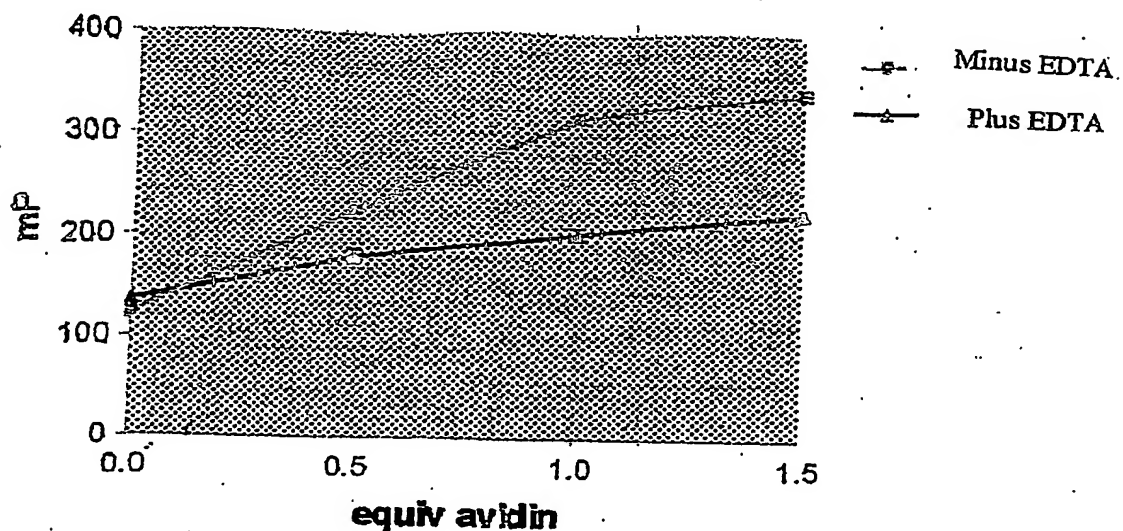


Mode of operation:	Linear
Extraction mode:	Delayed
Polarity:	Positive
Acquisition control:	Manual
Accelerating voltage:	20000 V
Grid voltage:	95%
Guide wire off:	0.05%
Extraction delay time:	200 msec
Acquisition mass range:	400 - 6000 Da
Number of laser shots:	100/spectrum
Laser intensity:	1605
Laser Rep Rate:	20.0 Hz
Calibration type:	External - D:Voyager\data/cal2_sept4.cal
Calibration matrix:	6-Cyano-4-hydroxycinnamic acid
Low mass gate:	400 Da
Orbitrizer start time:	12.814
Bin size:	2 m/z
Number of data points:	16059
Vertical scale:	1000 mV
Vertical offset:	0%
Input bandwidth:	160 MHz
Sample well:	51
Plate ID:	RB109FEB21
Serial number:	1197
Instrument name:	Voyager-DE
Plate type filename:	CAVOYAGER1100 well plate.apl
Lab name:	PE Biosystems
Absolute x-position:	1168.12
Absolute y-position:	22287.5
Relative x-position:	-421.377
Relative y-position:	380.011
Slide in spectrum:	100
Source pressure:	3.391e-005
Mirror pressure:	0
TC2 pressure:	0.01269
TIS gate width:	30
TIS flight length:	840

Acquired: 13/02/00, September 12, 2001
 I:\Biosystems\Releases\SVR\HIST-20a 0002.dci

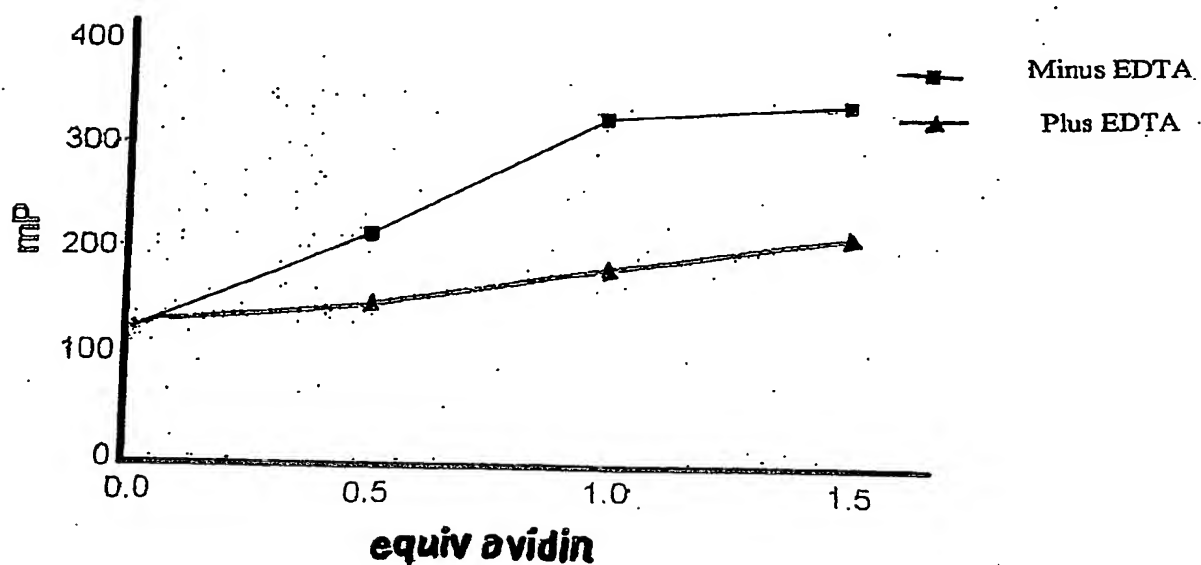
Figure 17 (b): MALDI-MS of Phosphorothiolated StarBright Green -PKC peptide target

Figure 18 (a) : Fluorescence Polarization of SBO-PKC-Ser-OPO2-S-BMCC-LC-Biotin after the addition of Avidin



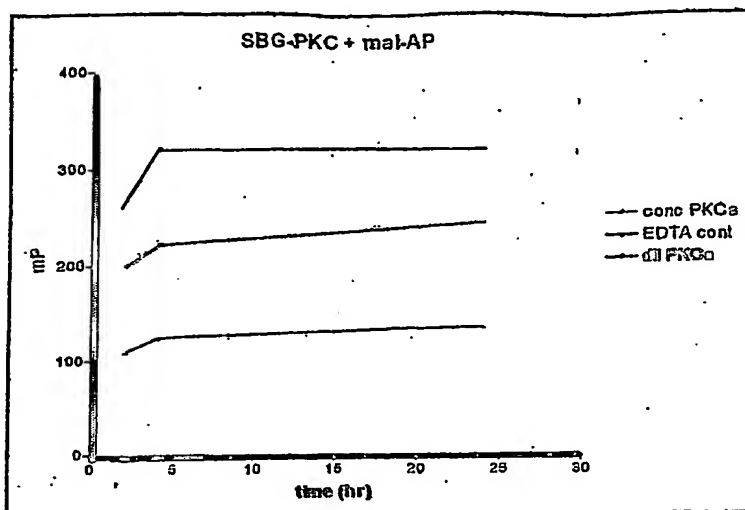
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Figure 18 (b) : Fluorescence Polarization of SBO-PKC-Ser-OPO2-S-Iodoacetyl-LC-Biotin after the addition of Avidine



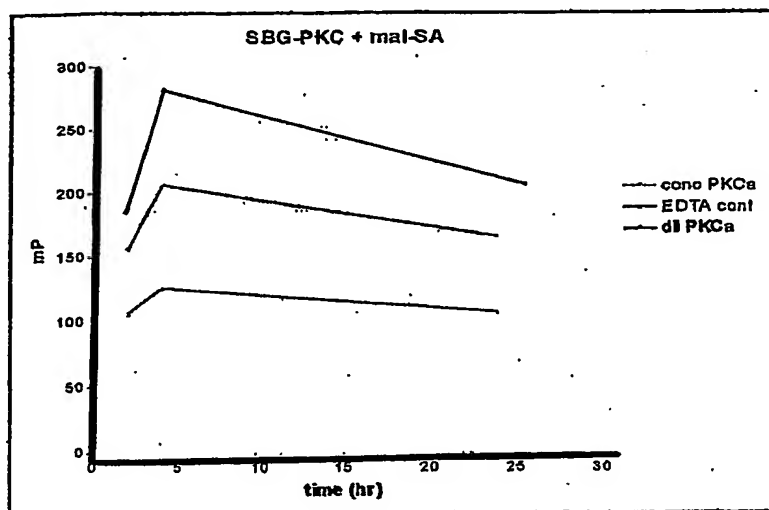
Fluorescence Polarization Using Large Molecules

(a)



time (hr)	conc PKCa	EDTA cont	dil PKCa
2	267	110	200
4	321	125	223
24	321	136	245

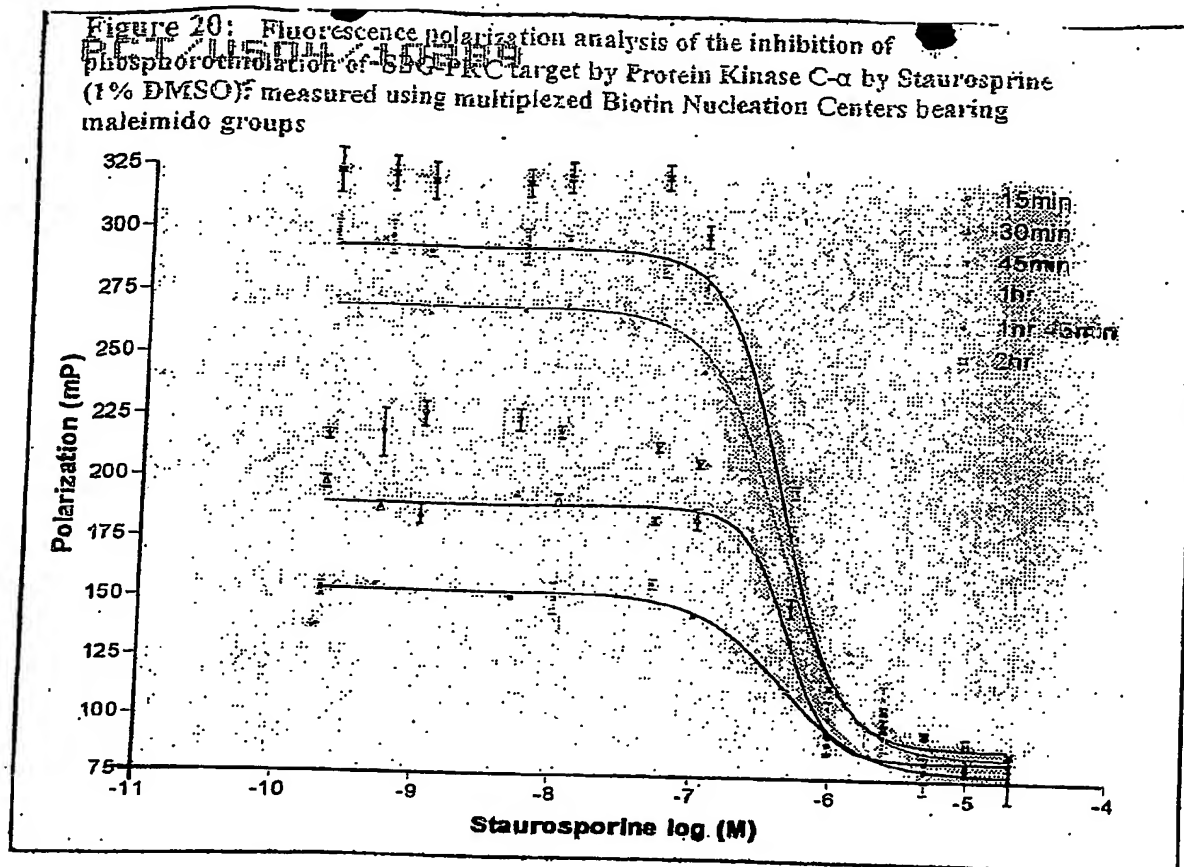
(b)



time (hr)	conc PKCa	EDTA cont	dil PKCa
2	187	106	156
4	281	126	207
24	213	107	166

Figure 19: Fluorescence polarization of analysis of phosphorothiolated SBG-PKC after the addition of multiplexed Nucleation centers comprised of Alkaline Phosphatase, figure (a), and Streptavidine, figure (b), bearing multiple maleimido groups capable of reacting with the phosphorothiolated peptide described in figure 13.

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EC50	4.39E-07	4.76E-07	4.40E-07	4.01E-07	4.16E-07	4.39E-07
KI	1.26E-07	1.36E-07	1.26E-07	1.15E-07	1.19E-07	1.26E-07

Fluorescence Polarization (mp)

15min	30min	45min	1hr	1hr45	2hr
67	78	85	82	85	86
80	78	83	77	81	89
87	69	85	74	83	98
87	98	96	101	105	106
93	85	94	86	101	108
113	111	134	133	108	113
142	143	188	179	174	193
154	158	181	184	283	294
143	158	189	183	282	317
150	149	193	193	288	315
151	152	180	188	300	324
155	152	185	189	291	311
155	149	189	184	289	328
149	154	183	191	302	313
74	78	71	83	291	326
152	145	180	191	75	86
		212	210	84	86
				282	319

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